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TITLE: Formulated Delivery of Enzyme/Prodrug and Cytokine Gene Therapy to Promote Immune Reduction of Treated and Remote Tumors in Mouse Models of Prostate Cancer

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14. ABSTRACT

Prostate cancer is the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery or radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatments are needed. We use gene therapy, alone and in combination with hormones called cytokines that stimulate the immune system. The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations. In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CD/UPRT)) is delivered to a cancer cell by a virus, or expressed by molecular engineering, so that harmless bacterial proteins are made. When followed by a pro-drug, 5 fluorocytosine (5FC), cancer cells that make CD/UPRT convert 5FC to a toxin that kills the original and neighbouring cells. This system works in slow growing tumors like prostate cancer. Killing the tumor cells attracts immune cells. We are identifying these and then delivering cytokine genes that attract more immune cells into the tumors. We will deliver the cytokine gene alone or with the suicide gene because in other studies, combination therapy works better.

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Formulated Delivery of Enzyme/Prodrug and Cytokine Gene Therapy to Promote Immune Reduction of Treated and Remote Tumors in Mouse Models of Prostate Cancer

Annual Report, January, 2006. DAMD17-02-1-0107

INTRODUCTION:

Prostate cancer (PC) is now the second highest cause of cancer death in men in Western society. Early disease is treatable by surgery and radiation, but once late stage disease becomes refractory to hormone removal, patient care is limited to pain management. New treatment strategies are needed. The **subject** of this work is a study of gene therapy, used alone and in combination with hormones called cytokines that stimulate the immune system. These therapeutic genes are delivered using lentiviral or adenoviral vectors, or by stable transfection into prostate cancer cells. *The concept is that delivering a cell-killing agent to an accessible tumor, coupled with help from the immune system can promote tumor reduction both at the treatment site and at remote locations.* In this therapy, a gene (a fusion of cytosine deaminase and uracil phosphoribosyltransferase (CDUPRT)) is delivered to a cancer cell so that harmless bacterial proteins are made. When a pro-drug, 5 fluorocytosine (5FC), is then given, cancer cells producing CDUPRT convert 5FC to a toxin that kills the original cell and others nearby. This strategy is suitable for slow growing tumors like PC. Killing the tumor cells attracts immune cells. The **scope** of the work involves preparation of the gene vectors, optimizing the conditions required for delivering the genes of interest by transfection or by using viral vectors, and identification of the immune cells that infiltrate the tumor when gene therapy is used. We are then using cytokine genes delivered into the tumor to attract more immune cells to this site. We have compared the effects of delivering the cytokine gene therapy alone, the suicide gene therapy alone, or a combination of both into mice that carry a murine prostate cancer cell line, RM1 cells, grown in the prostate. We predict that the combination therapy should interfere with the growth of the cancer cells in the prostate and should also cause a reduction in the number and extent of tumor cells that grow in the lung after introduction into the mice via intravenous injection. This work should pave the way for clinical trials of combination therapy involving suicide gene therapy and cytokine gene therapy given together into the prostate of men with PC.

BODY:

New cell lines have had to be prepared for the studies described below. In addition, we have had to prepare and characterize both plasmids, adenoviral and lentiviral vectors containing the genes of interest for delivery into PC. Not only have we manufactured the plasmids and recombinant viral vectors for delivery of the genes of interest, but we have also prepared stable transfectants from a murine PC cell line, the RM1 line, kindly provided by Dr T Thompson, Baylor College, Texas. The use of transfected cell lines will allow us to generate a maximum effect *in vivo*, and we will then be able to compare the possibilities that can be generated using a viral vector as the delivery vessel. In the first instance, the work was based on our previous studies that showed that the gene, purine nucleoside phosphorylase (PNP), could be used for gene-directed enzyme prodrug therapy (GDEPT) directed against PC (Martiniello *et al.*, 1998; Martiniello-Wilks *et al.*, 2002; Voeks *et al*., 2002a). However, due to problems with intellectual property, we were no longer able to use this gene, and moreover, workers from CSIRO decided not to be involved in the ongoing work. Instead, we have recruited Dr Aparajita Khatri, PhD (starting August, 2003) Dr Bing Zhang, PhD (started November, 2003, but left June 2004) and Ms Eboney Doherty, Bsc. Hons, who started in July, 2003. Unfortunately, Dr Zhang left us after 6 months for unforseen family reasons, and Ms Doherty left to study medicine. We subsequently recruited Ms Jane Chapman for technical help.

She started work in April, 2004). We have recently recruited Dr Yasmin Husaini to continue the work. Instead of using PNP, we have chosen the fusion gene, CDUPRT for the following reasons: CD converts 5 fluorocytosine to 5 fluorouracil, whose metabolites, 5-fluoro-2'-deoxyuridine 5'monophosphate (5FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) damage DNA and RNA respectively. The rate-limiting step in the generation of 5FdUMP and 5FUTP is the formation of an intermediary metabolite, 5-fluorouridine mono-phosphate (5FUMP). 5-FUMP is only produced after a series of catalysed enzymatic reactions. This can be circumvented by the ability of UPRT to convert 5FU directly to 5FUMP thereby leading to more efficient production of anti-tumor metabolites, 5FdUMP and 5FUTP (Tiraby *et al*., 1998). UPRT sensitizes cancer cells to low doses of 5FU (Kanai *et al*., 1998), and when used in conjunction with CD and 5FC in GDEPT, was more effective than CD-GDEPT alone against colon cancer (Koyama *et al*., 2000; Chung-Faye *et al*., 2001) and glioma (Adachi *et al*., 2000) *in vitro* and *in vivo*. There are no reports of this combination (CDUPRT) being used against PC, making our application novel. Thus *drugs generated by CDUPRT can kill both dividing and non-dividing cells*. This is important in PC, where the percentage of dividing cells is low. Moreover, *metabolites of 5 fluorocytosine can produce a local bystander effect* (Adachi *et al.*, 2000; Pierrefite-Carle *et al.*, 1999) and finally, *CD-GDEPT has been shown to generate a distant bystander effect* against colon carcinoma of the liver that was largely mediated by natural killer cells (Pierrefite-Carle *et al*., 1999).

During the last 12 months, we have made lentiviruses expressing CDUPRT, but have experienced difficulty in making sufficient virus for *in vivo* experiments. We have therefore implanted RM1 PC cells stably transfected with CDUPRT in the prostate of C57BL/6 males, and treated the mice with 5FC to test proof of principle of the CDUPRT/5FC GDEPT in our model system. We have done this is in the presence and absence of plasmids expressing mIL12 and mIL18, either singly or in combination in order to assess whether immunotherapy is synergistic with GDEPT. The results are detailed below.

DOD Alternate: The work was late in starting because of the intellectual property considerations, and the changes that were necessary to the program. The new program Statement of Work, accepted by the DOD, was shown in Appendix 1 of our Annual Report for 2003 (January, 2004). New staff were recruited, as described above, and because of this, the work could not be commenced until July, 2003. Unfortunately, we again lost staff for various reasons, and had to begin recruitment again. The current report represents a total of 2 years of work only. **We thus request a no-cost extension until the end of 2006 to complete our studies.**

Task 1:
GDEPT alone. Assess the ability of lentivirus expressing GDEPT (based on the fusion gene, cytosine deaminase/uracil phosphoribosyltransferase (CD/UPRT) to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 1-12)

a) Prepare recombinant lentivirus
Methods and Results:
Plasmid preparation and characterization: CDUPRT was obtained in the pORF-codA::upp plasmid from Invivogen (San Diego, CA, USA). The CodA::upp(CDUPRT) gene was excised from this plasmid using the *NcoI* and *NheI* restriction enzyme sites and ligated into complementary sites in the pVITRO2-GFP/LacZ expression plasmid (**See Jan 2004 report, Appendix II, Figure 1**). pVITRO2-GFP/LacZ, which also contains the genes for the jelly fish green fluorescent protein, GFP, and for the bacterial enzyme, βgalactosidase, LacZ, that can be used as reporter genes to monitor the progress of the preparation of plasmids, was used as a control vector and cell line throughout these experiments and provided the backbone plasmid for

insertion of all genes of interest. The resulting pVITRO2-GFP/CDUPRT plasmid was characterized for the presence of the genes of interest by restriction enzyme digestion using *NcoI* and *NheI* (**Jan 2004 report, Appendix II, figure 1**).

***Virus preparation and characterization**:*
The recombinant lentiviruses, Lenti.CMV.CDUPRT (GDEPT) and Lenti.CMV.GFP were constructucted for use in this study. The GFP expressing virus serve as a control virus for all the downstream experiments.
The virus construction is being performed according to the instructions in the ViraPower™ lentiviral expression system kit (Invitrogen, California, USA). The general strategy involves co-transfecting the pLenti-based expression vector with the packaging mix into the 293FT cell line (Invitrogen) to produce a lentiviral stock, which is then purified by ultracentrifugation. The purified stocks are then titrated for use in the proposed experiments.

Construction of pLenti.CDUPRT expression construct:
Gateway technology (Invitrogen) is based on the capacity of a site-specific recombination system to facilitate the integration of the bacteriophage lambda into the *E coli* chromosome and the switch between the lytic and lysogenic pathways (Ptashne, 1992); this system, modified to improve specificity and efficiency (Bushman *et al.,* 1985), was used to construct the expression clone, as described in January 2005 report. The entry clone containing the gene of interest flanked by *att* sites was constructed, and recombined with the destination vector (pLenti6/V5-DEST; Invitrogen) using the Clonase enzyme mix (Invitrogen) in an *in vitro* recombination reaction to yield the recombinant lenti-expression vector expressing the transgene for generation of the recombinant virus.

Step 1: Construction of the entry vector containing CDUPRT or GFP genes (pENTR1A-CDUPRT; pENTR1A-GFP): This was described in detail in our January 2005 report. PCR amplified CDUPRT or GFP genes were cloned in the entry vector (pENTR 1A; Invitrogen) to generate *pENTR-CDUPRT and pENTR-GFP vectors* (Jan 2004 report, Appendix II**, Figure 2A**). To ensure the fidelity and integrity of the amplification, *Pfu* TURBO DNA polymerase (Stratagene; California, USA) was used for the PCR amplification. Primers were designed (January 2005 report, Appendix IV) as described in January 2005 report. The pENTR.GFP and pENTR.CDUPRT constructs were analysed using the restriction digests (January 2005 report, Appendix II, **Figure 1**) and subsequently, the viruses rescued using these constructs were function tested (see below, step 3).

Step 2: Recombination of pENTR-CDUPRT or pENTR-GFP with the destination vector containing the Lentiviral elements (pLenti6/V5-DEST):
The details are available in our Jan 2005 report. Briefly, 300 ng of entry clone was recombined with 300 ng of destination vector, pLenti6-V5DEST (Invitrogen) *in vitro* using LR Clonase enzyme mix (Invitrogen) LR recombination mix was transformed into *E.coli* TOP10 and clones were screened for those positive for pLenti6/V5-CDUPRT or pLenti6/V5GP using appropriate restriction endonucleases (January 2005 report, Appendix II, **Figure 1A, 1B**). DNA from the positive clones (pLenti6/V5-CDUPRT) was propagated in HB101 strain of *E coli* for use in rescue of the recombinant viruses.

Step 3: Rescue of the Lent.CDUPRT and Lent.GFP using 293FT cells:
The purified DNA (representing the transfer vectors) was then used to rescue respective viruses using a four-plasmid transfection system (according to the instructions from the suppliers). The transfer vectors were co-transfected into 293FT cells (Invitrogen) with the packaging mix of three

plasmids, supplying viral proteins in *trans* for generation of the transgene containing Lentiviral particles (Naldindi et al., 1996). Transfection was optimized, by *Lipofectamine based* and *Calcium Phosphate based transfection (CaP)*, as described in the January 2005 report, since its efficiency dictates the viral yield. Optimization was done using pLent.GFP, as GFP expressing cells can be monitored by UV microscopy and flow cytometry, thus allowing accurate analysis of data, both qualitatively and quantitatively. Viral yields of up to $1.2x10^7$ transducing units/100mm dish were obtained after further optimization of this method (data not shown).

Functional test of Lent.GFP: The virus containing supernatants were analyzed by assessing the transduced 293 cells for GFP gene expression 72 h later, by UV microscopy and flow cytometry. These data showed that LentGFP is functional (details in Jan 2005 report).

Functional test of Lent.CDUPRT on RM1 cells: The Lent.CDUPRT was shown to be functional when tested on the 293A cells (January 2005 report, Appendix II, **Figure 3**). Next we tested the function of the virus on murine prostate cancer RM1 cells to be used in this study. Our earlier experiments using supernatant of unknown viral titre (Jan 2005 report, Appendix II, **Figure 3**); we now attempted to test the efficacy of the Lent.CDUPRT in killing tumor cells using supernatants of known viral titre. Parental RM1 tumor cells were plated in a 96 well plates at $5x10^3$ cells in 200μL complete DMEM medium/well. The next day, cells were infected with Lent.CDUPRT and Lent.GFP (control) viruses at three different mois (0.1, 1 and 10), then treated with 1mM 5FC for 48 h and analyzed for proliferation using WST1 reagent. Absorbance was measured at 450 nm. RM1 cells transduced with Lent.CDUPRT with no 5FC and RM1cells transduced with the control virus Lent.GFP in the presence of 5FC served as the controls. At an moi of 10, Lent.CDUPRT in the presence of 5FC killed 52% cells as compared to Lent.CDUPRT without 5FC or Lent.GFP in the presence of 5FC (January 2006 report, Appendix II, **Figure 1**). Our earlier experiments showed that only 10% of the RM1 cells were transduced when infected with Lent-GFP at an moi of 10tu/cell. Since ~52% of the cells were killed, this suggested that the local bystander effect may be involved.

While good titres (up to 10^8 tu/dish) were obtained initially (Invitrogen kit) at a smaller scale (1x100mm dish), we were unable to obtain optimal titres by large scale preparation for use in the *in vivo* experiments. To conduct one *in vivo* experiment involving 60 mice, at least $1x\ 10^{10}$ transducing units (tu) of the virus is required. Despite several attempts, currently we are only able to get $1\text{-}4x10^8$ tu from one large-scale preparation (20-40x100mm dishes see Table 1).

Table 1: Yield of virus from large-scale preparations

Virus	Titres tu/ml (~3mls each)
1. Lent.GFP Lot #1	$6x10^7$
2. Lent.GFP Lot #2	$6x10^7$
3. Lent.GFP Lot #3	$1.06x10^7$
4. Lent.CDUPRT Lot #1	$2.6x10^7$
5. Lent.CDUPRT Lot #2	10^7
6. Lent.CDUPRT Lot #3	$1.2x10^7$
7. LentCDUPRT Lot #4	$2x10^5$ (the cells did not survive the transfection)
8. Lent CDUPRT lot#5	10^6
9. LentCDUPRT Lot# 6	$1.2x10^6$

Note: Preparation and titration of each viral stock takes 3-4 weeks.

Optimization experiments are currently underway to further improve the viral titres. Large-scale preparation of recombinant lentiviruses involves transfection of 20 x 100 mm dishes of 293FT cells with the transfer plasmid containing the CDUPRT or GFP genes and the packaging mix containing the three other plasmids (see reports# 2004 and 2005 for details). The virus us purified from supernatants harvested 48 and 72 h post transfection by pelleting via ultra-centrifugation at 50,000 g for 2.5 h. We have found that we lose ~2-6 fold of the virus during the ultracentrifugation process, but this is within the expected range according the experience of other researchers. Viral titres are reliant on the efficiency of transfection which in turn depends upon:

- The quality of the 293FT cells. While we used early passage cells it is possible that sometimes due to over-confluency the acidic media might have altered the cells such that their transfectability was compromised (as advised by the technical support from the suppliers).
- The quality of the packaging mix (some of the lots supplied contained the resin from the purification columns and this affected the viral yields).

We are currently trying to optimize conditions on the basis of these facts in order to procure sufficient virus to conduct our *in vivo* experiments.

TASK 1b. Establish conditions for implanting TRAMP-C1 cells subcutaneously in transgenic TRAMP mice.

A relevant preclinical model that represents all stages of the human prostate cancer (PC) is required to evaluate potential therapies. Although commonly used, transplantable syngenic or xenogenic murine models do not emulate the considerable biological and technical challenges inherent in cancer treatment. The transgenic adenocarcinoma of the mouse prostate (TRAMP) model closely mimics early stages of the human disease. However, this model does not adequately represent late stage androgen-independent, metastatic cancer, and the timing to cancer is slow. We have therefore been working with TRAMP cell lines to circumvent this problem. As described in the 2005 report, we established that implanted TRAMP-C1 tumor cells in TRAMP (transgenic adenocarcinoma mouse prostate) mice did not grow until after 2 months, when they formed poorly differentiated prostate cancers, with loss of glandular architecture (Jan 2004 report, Appendix II, **Figure 3**).

Development of the TRAMP mouse model to represent different stages of prostate cancer

In order to conduct these experiments in TRAMP transgenics, the model has to be fairly consistent. We are currently breeding these mice at Biological Resource Centre, UNSW, available to us. However, recently we have faced a number of problems. The mice did not breed well (litter size has dropped to between 2-4), compromising the number of TRAMP positive male mice available (25% of a litter). Further, we found that the mice did not develop tumors at the time anticipated (~28-30 wks) with a fall in incidence to <40%. These problems further enhanced the already difficult logistics of conducting such complex experiments in a transgenic model. In addition, although reported by others (Greenberg *et al*., 1995; Gingrich *et al*., 1996) we have failed to find consistent metastases in these mice, and those we've seen have been limited to rare lymph node metastasis (Voeks *et al*, 2002b). Hence, to improve the logistics of experimental work and to broaden the model to represent late stage PC we have derived hormone refractory prostate cancer (HRPC) cell lines and characterized them *in vitro* and *in vivo* after implantation in syngeneic TRAMP null and C57BL/6 mice. The growth of TRAMP tumor derived parentals in the immunocompetent TRAMP null and C57BL/6 mice is well characterised in our laboratory (Voeks *et al*., 2002b). The experimental description and the results from this study are reported below.

Characterization of HRPC TRAMP cell lines:

This section of the work has been performed by PhD student, Varinder Jeet.

Three new HRPC cell lines were established from androgen dependent TRAMP cell lines TRAMP-C1 & TRAMP-C2 using both *in vitro* (depletion of androgen from medium) and *in vivo* methods (growth in female mice) (see January 2005 report). These cell lines, TC1-T5, TC1-F1 & TC2-T5 were derived from parental cell lines TRAMP C1 & TRAMP C2 respectively in our laboratory. They have been characterized as described below.

Methods & Results:

Task 1b.i Growth rates of different PCa cell lines *in vitro*:

a. Trypan blue exclusion: Cells were seeded at $1x10^5$/well in triplicate in a 24 well plates. Growth was measured by counting the viable cells via trypan blue exclusion of dead cells at 24, 48 and 72 h. *The results showed that TC1 and TC2 derived TC1-T5 & TC2-T5 cells grew relatively faster than the parent lines (Table 2).* The growth of these cell lines did not appear to be contact inhibited.

Table 2: Doubling times of TRAMP cell lines *in vitro*.

Cell Line	**Doubling Time (Hours)**
TC1	24.5
TC1-T5	22
TC1-F1	25.5
TC2	26
TC2-T5	21.5

b. Evaluation of anchorage independent growth: The capacity of a cell line for anchorage independent growth is indicative of its tumorigenicity. This was evaluated using the soft agar colony count assay. Cells were seeded at $5x10^4$/well in triplicate. Colonies (>20 cells) were counted after 12 days. There was a significant difference in the growth of these cell lines in soft agar (January 2006 report, Appendix II, **Figure 2**), with TC1-T5, TC2-T5 & TC1-F1 being able to form more colonies than the parental lines, TC1 and TC2, indicating their potential for increased tumorigenicity and invasiveness.

Task 1b. ii. Evaluation of the androgen-dependence of different TRAMP cell lines *in vitro*:

a. Evaluation of AR mRNA expression:

Androgen receptor (AR) is an intracellular membrane receptor, which has been shown to be critical in the development of hormone refractory prostate cancer (Furutani *et al.*, 2005). AR gene expression at mRNA level was determined by semi- quantitative RT-PCR and real time RT-PCR studies.

RNA extraction: Total RNA was extracted using tri-reagent (Molecular research centre, TR118) according to manufacturer's instructions (January 2006 report, Appendix II, **Figure3A**). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control.

Reverse Transcription (Ist strand cDNA synthesis): The cDNA was synthesised from 5μg of total RNA using Revert Aid M-MuLV reverse transcriptase (Fermantas, Aus).

Primers used for AR-PCR:

AR forward [5'-TGCTGCTCTTCAGCATTATTCCAGT-3'] (Foster *et al*., 1997)
AR reverse [5'-CCAGAAAGGATCTTGGGCACTTGC-3'];
GAPDH forward [5'-CCCATTGTGCTGTAGCCGTA-3']
GAPDH reverse [5'-AAGGGCTCATGACCACAGTC-3'].

For semiquantitative RT-PCR, multiplex PCR reaction involved denaturation at 94^0 C for 30 sec followed by annealing at 64^0 C for 30 sec and finally the elongation at 72^0 C for 2 min for 28 & 27 cycles, separately. The reaction was hot started (94^0 C for 5 min) and terminally extended at 72^0 C for 10 min. The results indicated a significant decrease in AR expression in derived cell line TC2-T5 compared to the parental TC2 (January 2006 report, Appendix II, **Figure 3B**).

Quantitative real time RT-PCR studies were done to evaluate the expression of AR by using SYBR green qPCR KIT (Invitrogen, Platinum SYBR green qPCR supermix UDG, 11733-038, Aus) with optimized concentrations of specific primers. Reaction conditions were set similarly to the RT-PCR, however changes were made to the times of different cycles according to the supplier's recommendations. Melting curves were generated after amplification to check PCR specificity. Amplicon specificity and reaction specificity were further confirmed by electrophoresis using 2% agarose gel (January 2006 report, Appendix II, **Figure 3C**). The changes in florescence of SYBR green dye in each cycle were monitored by the Rotor Gene system (Corbett Research, Aus), and the threshold cycle (Ct), which is defined as the cycle number at which the amount of amplified target reaches a fixed threshold, was obtained for each gene. Based on the Ct values of each gene for the derived cell lines TC2-T5 and parental line, TC2, relative ratios of GAPDH to AR and uPA were calculated (Table 3). The results indicated a significant decrease in the expression of AR in the derived cell line TC2-T5 compared to the parental TC2. Similar experiments will be performed for the other cell lines.

Table 3 Real time quantitative analysis of AR

Ratio (Crossing threshold (Ct)	TC2	TC2-T5
CtAR/ CtGAPDH	1.8	0.7

b. Evaluation of AR protein expression by immunohistochemistry:
The protein expression of AR in TC1 and TC2 parental and derived cells/ tumour sections was determined by immunohistochemical analysis of cells grown *in vitro*. Cells ($1x10^5$/well) were seeded in a four-chamber slide for each cell line and grown for 24h. Slides were then fixed with cold acetone for 2 min, quenched with hydrogen peroxide (0.03%) to block endogenous peroxidase activity and blocked with avidin, biotin and 2% IgG free BSA (10 mins each) to avoid non-specific binding. They were then incubated overnight at 4^0 C at with anti-AR antibody (Santa Cruz AR (N-20): sc-816; diluted 1:50), washed and incubated with rabbit-anti-mouse antibody (BA-1000, Vector Laboratories, diluted 1:200) for 30 mins, washed and counterstained with hematoxylin. AR expression was negative in the derived cell lines, TC1-T5, TC2-T5 & TC1-F1, whereas heterogeneous AR positive and AR negative cells were seen in the parental lines, TC1 and TC2 (January 2006 report, Appendix II, **Figure 4A**). Sections from paraffin-embedded tumors derived from *in vivo* growth of the different cell lines were also evaluated for AR status. Optimization of staining was done and microwave (100^0 C) and pressure cooker based (125^0 C) methods of antigen retrieval were compared. The pressure cooker based antigen retrieval was

successful. The results (January 2006 report, Appendix II, **Figure 4B)** indicated that parental TRAMP C1 show heterogenous staining for AR, whereas that in TC1-F1 and TC2-T3 tumors was weak or negative, respectively.

Task 1b.iii Evaluation of E-Cadherin expression by TRAMP cell lines *in vitro*:
E-Cadherin is a transmembrane glycoprotein that mediates cell-to-cell adhesion. The loss of its expression has been associated with metastases and poor prognosis in invasive cancer (Calvisi et al., 2004; Rakka et al., 2005). The TRAMP cell lines grown in chamber slides were assessed by immunohistochemistry, using the primary antibody (BD E-Cadherin, 610182, diluted 1:5000). E-Cadherin expression was found to be very low in TC1-T5 and TC1-F1 cells compared with the parental TRAMP C1 cells, indicating their potential for increased invasiveness (January 2006 report, Appendix II, **Figure 5**). In contrast, TRAMP TC2 derived TC2-T5 showed strong E-cadherin staining. This is not unexpected as other studies have shown that loss of E-cadherin expression is not always associated with increased invasiveness in cancer cells (Shimoyama et al., 1989).

Task 1b. iv Evaluation of uPA mRNA expression:
Urokinase type plasminogen activator (uPA) is highly over-expressed in the invasive tumour microenvironment of many human cancers and has been validated as a diagnostic and prognostic marker for prostate cancer, where its increased expression is directly correlated to advanced stages (Andreason *et al*., 1997; Forbes *et al.*, 2004; Usher *et al.*, 2005). Hence quantitative real time RT-PCR analysis was performed to determine the expression of uPA in derived cell line TC2-T5 compared to the parental TC2 (Same reaction conditions were used as described above). Primers used were:
uPA forward [5'-ACAGGGAAGACAGCCTGGCCTA-3']
uPA reverse [5'-CCCAGCTCACAATCCCACTCAG-3']
Relative ratios of Ct values of GAPDH: uPA were calculated, and found to be similar for both cell lines (1.2 for TC2 cells and 1.3 for TC2-TC5 cells (January 2006 report, Appendix II, **Figure 3C**). The other cell lines are currently being analysed for uPA expression.

SUMMARY:
We have derived new cell lines from TRAMP C1 and C2, which appear to be HRPC cells, possibly due to loss of expression of AR. We believe that these lines were selected from the original parental populationwhich were heterogeneous with respect to AR expression. These derived TRAMP cell lines are capable of increased anchorage independent growth compared with the parental lines, suggesting that they show increased invasive/tumorigenic potential; this is supported generally by their loss of expression of E-cadherin. Preliminary experiments investigating the growth rates o these cell lines in TRAMP null mice showed that these cells grew much faster ranging from 2wks to 4wks to reach 6mm compared with 6wks in case of parental cell line (January 2005 report, Appendix II, Figure 4). Further characterisation of these cell lines for various markers associated with late stage metastatic prostate cancer and determination of their *in vivo* growth characteristics is currently underway. If found to be metastatic and androgen independent *in vivo*, this could potentially expand the TRAMP model to represent late stage androgen independent, metastatic disease.

TASK 1c Optimize dose of virus needed to establish GDEPT in orthotopically implanted RM-1 tumors when formulated with plasmid.

Preliminary experiments were necessary as detailed in the January 2005 report. We determined that C57BL/6 mice could tolerate 5FC at a dose up to 500 mg/kg/mouse, given intraperitoneally (ip) every day for 13 days without systemic toxicity as observed by serum analysis (See January 2004 report, Table 1) or by histological examination of major organs (January 2004 report, Appendix II, **Figure 4**).

We also stably transfected RM-1 cells to express green fluorescence protein (GFP) and CDUPRT (test) (known as RM1-GFP/CDUPRT) or GFP alone or GFP.LacZ (controls) (known as RM1-GFP or RM1-GFP/LacZ) as described in our January 2004 report, section c. The GFP was used as a marker to follow the cells *in vivo*, and to sort high expressers. This was to provide proof of principle of the CDUPRT/5FC GDEPT whilst the lentiviral vectors for delivery of CDUPRT were being prepared. Methods were developed to measure CDUPRT expression *in vitro* by testing the capability of the cell/ lysates to catabolize the prodrug 5FC to 5FU (by HPLC: Jan 2005 report, Appendix II, **Figure 5A**) and by examining the effects of CDUPRT expression in the presence of 5FC on cell proliferation (Jan 2004 report, Appendix 2, **Figure 7**). Experiments confirmed that homogenates from RM1-GFP/CDUPRT tumors grown *in vivo* expressed CDUPRT enzymic activity by the HPLC assay. The sensitivity of the HPLC-based assay was also evaluated and the minimum protein concentration at which the 5FC-5FU conversion was detectable by HPLC was *0.3 mg,* equivalent to *~2x10^6* cells *in vitro*.

iii) Optimization of viral dose for delivery of CDUPRT-GDEPT

This is in progress.

TASK 1d. Assess ability of optimal doses of CD/UPRT-GDEPT (and control plasmid) injected intraprostatically into RM-1 tumors together with systemic pro-drug (5 fluorocytosine, 5FC) treatment to suppress local prostate and metastatic (lung) tumor development. &
TASK 1e. Examine other tissues for signs of toxicity that might result from escape of the CD/UPRT GDEPT virus from the site of injection.

These tasks require completion of section c before the studies can be performed. However, we have implanted RM1 cells stably transfected with CDUPRT in the prostate and treated the mice with 5FC to test **proof of principle**. The data acquired are reported in our paper, Khatri et al. submitted for publication to J Gene Medicine (September, 2005), results pending. (Appendix III) We have demonstrated proof of principle that CDUPRT-GDEPT + 5FC kills RM1 prostate cancer cells when given into the prostate, and also inhibits the growth of pseudometastases in the lung. Both a local and a distant bystander effect occur.

TASK 1f. Identify using immunohistochemistry, the immune cell types infiltrating the prostate tumors.
These studies are also described in the paper, Khatri et al, submitted for publication to J Gene Medicine (January 2006 report, Appendix III). In summary, immune cells infiltrating the primary tumor include CD4 and CD31 positive T cells, F480 positive macrophages and AsialoGM1+ NK cells. In addition, there was a decrease in the vasculature to the tumor, and an increase in apoptosis as measured by Tunel assay

Task 2.
pCytokine work: Assess the ability of lipid-enhanced delivery of an murine IL-12 or IL-18 expressing plasmids (pCytokine) to suppress orthotopic and metastatic prostate cancer in the RM-1 model (Months 12-22)

TASK 2a: Prepare pCytokine constructs

This work was initiated and reported in our January 2004 report. Plasmids expressing the murine IL-12 or murine IL-18 cytokines were prepared as pVITRO2-GFP-Cytokine (murine IL-12 and IL-18) constructs (see Task 2, sections (i), characterized and in the case of the mIL18 construct, sequenced (Jan 2004 report, Appendix III). These were called pVITRO2-GFP/mIL12 and pVITRO2-GFP/mIL18 (Jan 2004 report, Appendix II, F**igures 13 & 14**).

To ensure that the RM1 prostate cancer cell lines would not be stimulated by exposure to these cytokines, they were tested for the presence of surface IL12 receptor (IL12R) and IL18 receptor (IL18R) by FACS analysis using suitable antibodies as described in Jan 2004 report, Task 2, sections (i) and (ii) and Jan 2004 report, Appendix II, **Figures 12 and 15**. The RM-1 cells did not express either IL12R or IL18R.

TASK 2b: Determine dose of pCytosine-construct plasmid DNA (0.1-1.0 g) which, when formulated with lipid and control virus leads to detectable expression of cytokine in orthotopically implanted RM-1 tumors by:
(i) Harvesting tumor cells, culturing and monitoring cytokine production by Western blot.

Whilst the lentiviral constructs expressing the cytokines were being prepared, we generated stable transfectants of RM1-GFP/IL12 and RM1-GFP/IL18 under hygromycin B selection (800 μg/mL) as described in the January 2004 report, under Task 2. Direct sequencing confirmed the appropriateness of the plasmid inserts. The same control cells as for RM1-GFP/CDUPRT, stably transfected to express GFP alone or GFP/LacZ, and known as RM1-GFP or RM1-GFP/LacZ (controls) respectively, were used. Again, GFP was used as a marker to follow the cells *in vivo*, and to sort high expressers as described previously (see Jan 2004 report, Appendix II, **Figures 5, 6, 13**). Western blotting was performed and showed that the mIL12 and mIL18 were being produced by the respective lines. A respresentative Western blot for mIL18 is shown in Jan 2006 report, Appendix II, **Figure 6**. We also developed a biological assay system using CTLL2 cells to measure the IL12 and IL18 production by RM1-GFP/IL12 and RM1-GFP/IL18 cells as described in our January 2005 report, under Task 2b. A paper describing this work has been prepared for submission to Journal of Immunological Methods, See Appendix IV. Our experiments have shown that:

1. *CTLL2 cells respond to both mIL12 and mIL18.*
2. *RM1-GFPmIL12 and RM1-GFPmIL18 secrete functional cytokines.*
3. *The proliferative response of the CTLL2 cells is further enhanced when the two cytokines are used in combination.*

(ii) TASK 2b: Measuring biological activity of secreted cytokine using a cytotoxic lymphocyte (CTL) bioassay.&
(iii) TASK 2b: Measuring cytokine mRNA production by RT-PCR.

This work has not been done. Instead, we have examined the effects of mIL12 and mIL18 on *in vivo* growth of RM1 cells by implanting the stably transfected RM1-GFP/mIL12 or RM1-GFP/mIL18 cells, as described in our January 2005, report (Table 4, and Appendix II, **Figures 16**

and 17). These experiments indicated that both the mIL12 and mIL18 were biologically active and secreted *in vivo* (both after subcutaneous or intraprostatic implantation), inhibiting tumor take and growth compared with control tumors (RM1-GFP/LacZ). The tumors that formed from RM1-GFP/mIL12 were highly necrotic and infiltrated with increased numbers of immune cells compared with control tumours, in particular NK (Asialo-GM1 positive cells, seen in clusters), both CD8 positive (few) and CD90 positive (especially around blood vessels and in haemorrhagic necrotic areas) and some F4/80 positive cells (see January 2005 report, Appendix II, **Figure 18**).

The following studies have not yet been undertaken, but instead, work has been done using RM1-GFP/CDUPRT cells implanted into the prostate of C57BL/6 mice, with mIL12 and mIL18 plasmids injected introtumorally; mice were treated with 5FC to elicit GDEPT or saline, ip, daily until euthanasia (please see below).

TASK 2c. Determine impact of administering 5-FC prodrug on immune cell recruitment into tumors following injection of cytokine gene plasmid/lipid/control virus administration &
TASK 2d. Determine the persistence of cytokine production by transfected tumors.

TASK 2e. Compare ability of transfected pCytokines (optimal dose complexed with lipid and control virus) to suppress orthotopic and metastatic RM-1 prostate tumor growth.
TASK 2f. Choose optimal cytokine gene system on basis of maximum suppression of tumor growth obtained.

TASK 3. **Combination therapy**: Assess the ability of delivery of a combined virus borne GDEPT and lipid delivered plasmid-borne cytokine gene therapy to suppress orthotopic and metastatic prostate cancer in RM-1 model and in TRAMP mice carrying sc TRAMP-C1 grafts. (Months 22-33)

a. Determine whether pCytokine-enhanced immune activity affects GDEPT.
b. Determine the effects of injecting lentivirus expressed GDEPT and pCytokine intraprostatically (using optimal doses of each component as revealed by Tasks 1A and 1B) on orthotopic tumor growth and metastases.

In vivo experiments

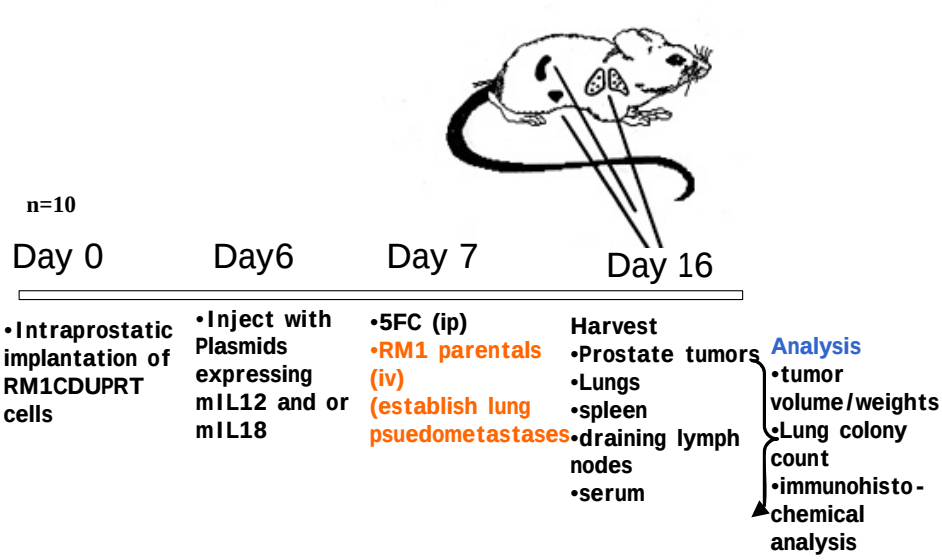
Efficacy of CDUPRT GDEPT in combination with pVITRO2.mIL12 and/or pVITRO2.mIL18 on the growth of intraprostatic RM1 tumors in C57BL/6 mice

Experimental Design:
In general, mice previously implanted in the prostate (iprost) with RM1-GFP/CDUPRT tumors (abbreviated RM1CDUPRT) were given intratumoral (i.t.) injections of either pVITRO2GFP.mIL12 or pVITRO2GFP.mIL18 or a combination of the two with and without 5FC. The plasmid pVITRO2GFP.LacZ was used as the control plasmid and RM1GFP.LacZ cells were used as control cells for these experiments. The experiment was performed as follows:

Day 0: Mice were implanted intraprostatically with 2.5 $x10^4$ RM1CDUPRT cells.
Day 6: Mice were given i.t. injections of either pVITRO2GFP.mIL12/ pVITRO2GFP.LacZ or pVITRO2GFP.mIL18/ pVITRO2GFP.LacZ or a combination of the two, pVITRO2GFP.mIL12/ pVITRO2GFP.mIL18 at the dose of 19μg of each plasmid /mouse.

Day 7: Mice were injected with 2.5 x10^5 RM1 parental cells intravenously (iv) to establish pseudometastases in the lungs. Mice were also given 5FC (500 mg/kg/mouse/day) or saline, intraperitoneally (ip) each day until necroscopy (day 16).
At necroscopy, mice were euthanased and tumors and other organs including spleen and draining lymph nodes were harvested; these were either formalin fixed or snap frozen (in liquid nitrogen) for histological and immunohistological analyses. Mouse sera were also harvested and stored at –80^{o}C for future analyses. The lungs were fixed in Bouin's reagent and colonies were counted as described in Martiniello-Wilks *et al*, 2005.

Experimental strategy:



The experiment was done in two parts due to logistical problems associated with large numbers of mice involved

The treatment groups were as follows:
<u>CDUPRT + IL12</u>

1. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.mIL12/ pVITRO2.GFP.LacZ @19µg/plasmid/mouse (total 38µg/mouse) with saline (ip,everyday until necroscopy).
2. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.mIL12/ pVITRO2.GFP.LacZ @19µg/plasmid/mouse with 5FC (ip,everyday until necroscopy).

<u>CDUPRT + IL18</u>

3. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.mIL18/ pVITRO2.GFP.LacZ @19µg/plasmid/mouse with saline (ip,everyday until necroscopy)
4. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.mIL18/ pVITRO2.GFP.LacZ @19µg/plasmid/mouse with 5FC(ip,everyday until necroscopy)

<u>CDUPRT + IL12 + IL18</u>

5. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP-IL12 and - IL18 @38µg/plasmid/mouse with saline (ip, everyday until necroscopy)
6. Mice implanted with RM1CDUPRT cells injected i.t. with with pVITRO2.GFP-IL12 and - IL18 @38µg/plasmid/mouse with 5FC (ip,everyday until necroscopy)

<u>LacZ+IL12+IL18</u>

7. Mice implanted with RM1LacZ cells injected i.t. with with pVITRO2.GFP-IL12 and -IL18 @38μg/plasmid/mouse with saline (ip, everyday until necroscopy)
8. Mice implanted with RM1LacZ cells injected i.t. with with pVITRO2.GFP-IL12 and -IL18 @38μg/plasmid/mouse with 5FC (ip,everyday until necroscopy)

Results:

The growth of RM1CDUPRT and RM1 LacZ tumours was reassessed in the prostate of C57BL/6 mice because the cell lines had to be rederived *in vivo*, due to possible Mycoplasma contamination. Both cell lines grew in a dose dependent manner, however the RM1CDUPRT cells grew more rapidly compared with the control cell line, RM1LacZ (Jan 2006 report, Appendix II, **Figure 7**). On the basis of these results, the dose of cells for iprost injection chosen was $2.5x10^4$ cells for RM1CDUPRT and $5x10^4$ for RM1LacZ cells.

Efficacy of CDUPRT GDEPT in combination with pVITRO2.mIL12 or pVITRO2.mIL18 on growth of intraprostatic RM1 tumors in C57BL/6 mice

There were no effects of any treatment on the growth of iprost tumors at necroscopy of the different groups of mice (Jan 2006 report, Appendix II, **Figure 8**). This result was surprising, especially given that CDUPRT/5FC GDEPT had previously been shown to diminish prostate tumor growth (see paper, Appendix III). A possible explanation is that the RM1CDUPRT cells were implanted at five fold the doses that we used earlier, based on the growth of the rederived cells (Jan 2006 report, Appendix II, **Figure 7**). Since the RM1-GFP/CDUPRT tumors grew to large volumes, their aggressive nature may have overcome the effects of therapy in the timeframe involved. We have repeated this experiment using a lower dose of RM1-GFP/CDUPRT cells and found an effect of GDEPT on tumor growth (see below).

Distant bystander effects

RM1 tumor pseudometastases in lungs were assessed. In contrast to the tumor growth in the prostate, the growth of RM1 lung colonies was GDEPT (CDUPRT/5FC) dependent in all treatment groups (Jan 2006 report, Appendix II, **Figure 9**). However, neither pVITRO2.mIL12 nor pVITRO2.mIL18 when used alone showed any additive or synergistic effects with CDUPRT/5FC GDEPT.

Efficacy of CDUPRT GDEPT in combination with pVITRO2.mIL12 and pVITRO2.mIL18 growth of intraprostatic RM1 tumors in C57BL/6 mice

As with the use of either plasmid alone, a combination of pVITRO2.mIL12 and –mIL18 with CDUPRT/5FC GDEPT had no significant effect on the growth of tumors in the prostate (Jan 2006 report, Appendix II, **Figure 10**). Again, we believe that any effects may have been masked by the aggressive growth of the tumors, and will be repeating this experiment with a lower dose of RM1-GFP/CDUPRT cells. A repeat of this work using a lower dose of RM1-GFP/CDUPRT cells in the prostate implantation has revealed that GDEPT is not affected by introducing p pVITRO2.mIL12 or –mIL18 alone, as described below (see Jan 2006 report, Appendix II, **Figure 16**).

Distant bystander effects

In contrast to the effects of each plasmid alone, the combination of pVITRO2.mIL12 and -mIL18 was extremely potent and dramatically diminished the lung colony formation, irrespective of the GDEPT (Jan 2006 report, Appendix II, **Figure 11A**). Whilst the local effects of either plasmid alone or in combination were absent, the distant bystander effects indicated that the treatments were effective, most likely through stimulation of an immune response. A comparison of treatments using RM1-GFP/CDUPRT and 5FC or saline with pVITRO2.mIL12 or

pVITRO2.mIL18 in combination with pVITRO2.LacZ (Control plasmid), or with the two cytokine plasmids is shown in Jan 2006 report, Appendix II, **Figure 11B.**

Studies of immune response.

Prostates were harvested at necropsy from each treatment group and snap frozen sections from 3 mice/group were immunostained for the presence of CD4+ and CD8+ T cells, macrophages and NK cells using antibodies and methods as described in the 2005 report. These preliminary data are summarized in Table 4; more mice will be examined each group to generate statistically significant results. There were several points of interest:

1. CD4 staining: Combining mIL18 + GDEPT (C) resulted in increased CD4+ T cells in the tumor compared to mIL12 + GDEPT (B) or GDEPT alone (A). Using both plasmids together caused significantly increased (3.5-fold) CD4+ cells with or without GDEPT and when RM1LacZ cells were used in combination with mIL12 + mIL18 (E) (Jan 2006 report, Appendix II, **Figures 12, 13**),the increase in CD4+ cells was higher than that seen with RM1CDUPRT cells (D). This effect was more acute when 5FC was also used. No significant differences were seen between saline and 5FC groups in all treatment groups. This finding suggests that CD4+ T cells are involved in the distant bystander effect seen when mIL12 and mIL18 were combined.

2. CD8A staining: Increased CD8+ cells were seen in tumors treated with mIL18 plasmid alone (C), and in those that received mIL18+ mIL12 (D and E), but not when mIL12 (B) or GDEPT (A) was used alone. There was a trend to enhancement of CD8+ T cell infiltration in the presence of GDEPT with 5FC in C, D and E compared to saline. This increase was most significant in E, suggesting greater immunogenicity of RM1-GFP/LacZ cells compared with RM1-GFP/CDUPRT cells, possibly due to a greater immune response to the LacZ gene product.

3. Asialo GM: There were no significant differences between NK cell infiltration seen in all treatment groups (Jan 2006 report, Appendix II, **Figure 14**). However a general reduction was observed in 5FC treated groups compared with saline treated controls in treatment groups A,C and D. The use of mIL18 plasmid alone + GDEPT (C) appeared to suppress asialo GM+ cell infiltration, but not when used in combination with mIL12 plasmid + GDEPT and RM1LacZ cells (E). When pVITRO2.mIL12 was used alone with GDEPT, there was no reduction in asialoGM+ cells, suggesting that the effects of mIL12 on NK cell infiltration are more potent than those of mIL18 alone in overcoming any immunosuppression generated by the GDEPT.

4. *F4/80 staining:* In contrast to the trends observed with CD4+ and CD8+ infiltration, macrophage infiltration was minimal in tumors treated with mIL18 plasmid (C) compared those treated with mIL12 plasmid (A) or GDEPT alone (B). The presence of CDUPRT/5FC GDEPT appeared to diminish the macrophage infiltration (A-D) compared to tumors from RM1LacZ cells (E) (Jan 2006 report, Appendix II, **Figure 15**). This may reflect the immunosuppressive nature of 5FU generation by the GDEPT. Infiltration was significant when RM1-GFP/Lac Z cells were used with the two IL plasmids (E), or when RM1-GFP/CDUPRT cells were injected with pVITRO2.LacZ (A), again supporting our hypothesis that the LacZ gene product may be immunogenic.

Table 4: Immunohistochemical analysis of immune cell infiltration in intraprostate tumors from different treatment groups.

	CD4^{+}		CD8^{+}		**F4/80^{+}**		**AsialoGM^{+}**	
	Saline	***5FC***	***Saline***	***5FC***	***Saline***	***5FC***	***Saline***	***5FC***
A:RM1-CDUPRT+pvitro2GFPLacZ	6.7	1.1	2.4	1.2	23.5	8.8	26.6	14.1
B:RM1-CDUPRT+pvitro2GFPLacZ+pvitro2GFPmIL12	1.6	1	2.7	1	15.2	11.6	21.4	21.1
C:RM1-CDUPRT+pvitro2GFPLacZ+pvitro2GFPmIL18	8.3	10.2	2.5	6.8	7.8	1	21.6	5.4
D:RM1-CDUPRT+pvitro2GFPmIL12+pvitro2GFPmIL18	18.7	10.6	8.6	9.4	24.2	4.9	22.9	11.1
E:RM1-LacZ+pvitro2GFPmIL12+pvitro2GFPmIL18	22.2	25.2	7.6	16.2	33.1	28.4	22.5	20.1

Numbers represent mean number of positive cells/high power field (x63) from 10 fields counted.

In conclusion, we have shown that the combination of mIL12 and mIL18 plasmids has synergistic immunostimulatory effects in preventing pseudometastatic growth in our model. The lack of efficacy against the local intraprostatic tumor growth may be overcome by using a lower dose of aggressive RM1-GFP/CDUPRT cells for implantation (please see below). Next, we will characterize the immune response generated by immunodepletion of CD4+, CD8+, and NK cells macrophages *in vivo*.

The above experiment was repeated for the following reasons:

1. To reconfirm the promising data showing the efficacy of combination of the two plasmids in inhibiting lung pseudometastases.
2. To re-evaluate the combination strategy, since
    a. None of the treatments had an inhibitory effect on the intraprostate tumor growth.
    b. While GDEPT led to inhibition of lung metastases, the combination of GDEPT with either mIL12 or mIL18 expressing plasmids did not lead to a greater distant bystander effect.

Experimental design: *Changes were made to the experimental design on the basis of the observations and results obtained in the previous experiment. The changes were as follows:*
Day 0: The mice were implanted intraprostatically with $9x10^3$ RM1-GFP/CDUPRT cells. It is possible that the tumour burden was too high in the previous experiments for the treatments to show efficacy on intraprostatic tumour growth in the timeframe involved. Hence in order to increase the therapeutic window the dose was reduced by ~3 fold.
Day 6: Mice were given i.t. injections of either pVITRO2GFP.mIL12 + pVITRO2GFP.LacZ, pVITRO2GFP.mIL18 + pVITRO2GFP.LacZ, or pVITRO2GFP.mIL12 + pVITRO2GFP.mIL18 at a dose of 17μg of each plasmid /mouse. The plasmid dose was reduced due to an insufficient amount of DNA available on the day of the experiment.

Day 7: Mice were also given 5FC (500 mg/kg/mouse/day) or saline, intraperitoneally until necroscopy (day 14). The iv implantations were postponed by a day so that we could evaluate the effects of treatments on the establishment and growth of the lung pseudometastases.
Day 8: Mice were injected with 2.5 $x10^5$ RM1 parental cells intravenously to establish pseudometastases.
At necroscopy, mice were euthanased and tumors and other organs including spleen and draining lymph nodes were harvested and were either formalin fixed or snap frozen (Liquid Nitrogen) for histological and immunohistological analyses. Mouse serum was also harvested and stored at –80^oC for future analyses. The lungs were fixed in Bouin's reagent and colonies were counted as described previously.
The treatment groups were as follows:

i. Mice implanted with RM1-GFP/CDUPRT cells injected i.t. with pVITRO2.GFP.mIL12/ pVITRO2.GFP.LacZ @17μg/plasmid/mouse (total 34 μg/mouse) with saline (ip, everyday until necroscopy).
ii. Mice implanted with RM1-GFP/CDUPRT cells injected i.t. with pVITRO2.GFP.mIL12/ pVITRO2.GFP.LacZ @17μg/plasmid/mouse with 5FC (ip, everyday until necroscopy).
iii. Mice implanted with RM1-GFP/CDUPRT cells injected i.t. with pVITRO2.GFP.mIL18/ pVITRO2.GFP.LacZ @17μg/plasmid/mouse (total 34 μg/mouse) with saline (ip, everyday until necroscopy)
iv. Mice implanted with RM1-GFP/CDUPRT cells injected i.t. with pVITRO2.GFP.mIL18/ pVITRO2.GFP.LacZ @17μg/plasmid/mouse (total 34 μg/mouse) with 5FC (ip ,everyday until necroscopy)
v. Mice implanted with RM1-GFP/CDUPRT cells injected i.t. with pVITRO2.GFP.LacZ @34μg/plasmid/mouse with saline (ip, everyday until necroscopy)
vi. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.GFP.LacZ @34μg/plasmid/mouse with 5FC (ip, everyday until necroscopy)
vii. Mice implanted with RM1CDUPRT cells injected i.t. with pVITRO2.mIL12 +pVITRO2.mIL18 @34μg/plasmid/mouse with saline (ip, everyday until necroscopy)
viii. Mice implanted with RM1CDUPRTcells injected i.t. with pVITRO2.mIL12 +pVITRO2.mIL18 @34μg/plasmid/mouse with 5FC (ip, everyday until necroscopy)
ix. Mice implanted with RM1LacZ cells injected i.t. with pVITRO2.mIL12 +pVITRO2.mIL18 @34μg/plasmid/mouse with saline (ip, everyday until necroscopy)
x. Mice implanted with RM1LacZ cells injected i.t. with pVITRO2.mIL12 +pVITRO2.mIL18 @34μg/plasmid/mouse with 5FC (ip, everyday until necroscopy)

Results:

The experiment had to be stopped on day 14 as some mice started to show signs of distress and loss of condition.

Effects of different treatments on growth of intraprostatic RM1 CDUPRT tumours

Unlike the previous experiment there was reduction in the growth of RM1-GFP/CDUPRT tumors in the presence of 5FC suggesting that the high implantation dose may have led to the lack of efficacy in the previous experiment (Jan 2006 report, Appendix II, **Figure 16)**. However, when CDUPRT-GDEPT was combined with either pVITRO2.mIL12 or pVITRO2.mIL18 treatments the efficacy was reversed. Interestingly, when GDEPT was combined with pmIL12 treatment the growth appears to be more in the 5FC treated group. The reasons for this are not clear and need further investigation. Finally when GDEPT was combined with both pVITRO2.mIL12 + pVITRO2.mIL18, there is a reduction in the growth of tumors compared with the saline controls. While not significant the trends were obvious in the GDEPT alone versus

GDEPT+pmlL12+mIL18 groups. It is not clear why the animals lost condition so early in this experiments. To investigate this we will analyse the blood serum harvested at the time of necroscopy for markers of liver and kidney function in different groups. This reduction in the therapeutic window may have affected the outcome of this experiment.

Effects of different treatments on growth of RM1 lung pseudometastasesRM1 tumor pseudometastases in lungs were assessed by counting number of tumor colonies. In contrast to the tumor growth in the prostate, the growth of RM1 lung colonies was GDEPT (CDUPRT/5FC) dependent in all treatment groups (Jan 2006 report, Appendix II, **Figure 17**). Interestingly, pVITRO2.mIL18 alone and in combination with -mIL12 and GDEPT reduced the lung colony formation but pVITRO2.mIL12 alone did not have much effect (Jan 2006 report, Appendix II, **Figure 17**). Although the local effects of either plasmid alone or in combination was not dramatic, the distant bystander effects indicated a distant bystander effect, most likely through stimulation of an immune response.

Studies of immune response.
Immunohistochemical analysis of tumour infiltrating immune cells was carried out in the Zn-fixed paraffin-embedded mouse tissue sections. Other tissue analysed were spleen and thymus as positive controls. Tissue sections were stained for CD4+ (T helper cells), $CD8a^+$ (Cytotoxic T lymphocytes), $F4/80^+$ (macrophages) and Asialo-GM^+ (NK) cells. For each type of immune cell analysis tumours from at least 3 mice from each treatment group were analysed. For each tumour section at least 4 different high power (x63) fields were scored and an average was taken. These data is not complete, as at least 10 high power fields need to be done for statistical significance(This is currently being done). These analyses were done blind.

CD4 (T helper cells) staining: The data obtained clearly showed an enhancement of CD4+ cells in GDEPT treated groups in comparison to saline controls in all treatment (Figure 18, panel A). Interestingly, the same pattern was observed with the non-GDEPT, RM1LacZ+pmIl12+ pmIL18 group. It is not clear why there is increased infiltration by CD4+ cells in 5FC group (group x). This correlated with the trends observed in growth of lung colonies in all treatment groups (Figure 17). The CD4+ infiltration was dramatically enhanced when GDEPT was in combination with mIL12 and/or mIL18. However, this enhancement was also observed to a lesser extent in RM1LacZ+pmIl12 and pmIL18 group, which could be attributed to the presence of the two cytokines.

CD8 (Cytotoxic T lymphocytes) staining: There was no significant infiltration by the CD8a + cells in any of the treatment groups. This negative result was valid as the staining was strongly positive in the mouse spleen and thymus. This was in contrast to enhancement of CD8a staining observed in the previous experiment. It is possible that infiltration by the CD8a+ cells requires a larger therapeutic window (> 1wk), which was reduced by 3-4 days in this experiment.

F4/80 (macrophage) staining: There was in general an enhancement of F4/80+ cells in all 5FC treated groups except for the non-GDEPT RM1LacZ.pmIL12+pmIL18 group. This was expected and correlated well with the trends observed with lung colony growth in the respective groups**.** Further, there was dramatic increase in the macrophage infiltration when GDEPT was combined with either or a combination of the two plasmids. This however was not reflected in the lung metastases data obtained from these treatment groups (Figure 17). Again, these results may be due to shorter therapeutic window in this experiment.

AsialoGM (NK cells) staining: The staining of the Zn fixed sections with this antibody lead to very high background and hence we were unable to score. This is currently being repeated with the frozen sections from tumors from each of the different treatment groups.

In conclusion, while the data obtained in this experiment are not conclusive, it clearly shows synergy between the GDEPT and the cytokine treatment. Unfortunately due to the loss of condition of the mice, the experiment had to be terminated prematurely and that possibly led to some of the difference in trends with respect to the Iprost and lung tumor growth in comparison with the previous experiments. We plan to repeat the same experiment using I.t injections of Adenoviral vectors expressing mIL12 and mIL18 (Note: we have already rescued and function tested these vectors). We hope to get more potent immunological effects using the immunogenic Adenoviral vectors than were obtained with the plasmids.

Analysis of apoptosis in different treatment groups was done using Caspase-3 assay. The results are tabulated in Table5.

Table 5: Caspase-3 assay

1		2		3		4		5		6		7		8		9		10	
IL12+CDUPRT				IL-18+CDUPRT				LacZ+CDUPRT				IL-12+IL-18+LacZ				IL-12+IL-18+CDUPRT			
5-FC		**Veh**		**5-FC**		**Veh**		**5-FC**		**Veh**		**5-FC**		**Veh**		**5-FC**		**Veh**	
413.2	3.4	411.2	11	419.2	8.8	414.2	10	417.2	5.8	418.2	13.9	425.1	8.5	421.2B	5.9	416.1	10	412.1	9.6
424.2	10.5	422.3	7.2	419.3	6.2	427.3	7.6	432.3	6.9	418.3	10.2	425.3	9.2	435.1	10	421.1B	7.9	412.2	6.9
431.2	6	423.1	8	429.1	14.6	439.2	7.3		*f*	423.2	9.1	426.3	9.4	438.1	8.1	436.2	8.2	420.3	242.1
average	6.63		8.73		9.87		8.30		6.35		11.07		9.03		8.00		8.70		86.20

Using a two-tailed T-test there is no significant difference between the 5-FC and Vehicle treated in different treatment groups. Further there weren't any significant differences observed in different treatment groups.

TASK 4. **Tissue slice work**: Assess the ability of lentivirus encoding green fluorescence protein (GFP) under a prostate directed promoter from Dr Paul Rennie, Vancouver to express GFP in human tissue slices. (Months 24-33).

We have recently acquired a Krumdeick Tissue slicer (Birmingham, Alabama) through an equipment grant (Rebecca L Cooper foundation) to conduct these experiments. Dr Khatri has recently finished the training necessary to use precision cut tissue slices cultured *in vitro* for assessment of transcriptionally targeted Lentiviral vectors (to be obtained from Dr. Paul Rennie). We are now in an excellent position to conduct this study ..

Task 5. Collate data, prepare reports and manuscripts (Months 33-36)
Some papers have been written (please see Jan 2006 report, Appendix III and Appendix IV). We are requesting a no-cost extension to complete the work. Our studies have been slowed down by the movement of staff and the necessity for recruitment for new staff.

KEY RESEARCH ACCOMPLISHMENTS:

- Established stably transfected murine prostate cancer lines from RM1 that express the transgenes and the reporter gene, green fluorescence protein: RM1-GFP/CDUPRT cell line; RM1-GFP and RM1-GFP/LacZ, RM1-GFP/mIL12 and RM1-GFP/mIL18 cell lines.
- Established and tested assay systems to measure expression of the transgene, CDUPRT *in vitro* and *in vivo*.
- Shown that CDUPRT-GDEPT + 5FC is associated with a ***local bystander effect***
- Shown that CDUPRT-GDEPT + 5FC given into the prostate is also associated with a ***distant bystander effect***, resulting in a reduction of the growth of pseudometastases of RM1 cells in the lungs of C57BL/6 mice, and with immune cell infiltration in the prostate
- Shown that CDUPRT-GDEPT +5FC effect is associated with infiltration of the tumors with immune cells, especially, macrophages, NK cells and CD4+ cells. Also the treated tumors show greater levels of necrosis and apoptosis, disrupted vasculature and enhanced proliferation in comparison to control tumors. *This is encouraging as it suggests further augmentation of the immune responses when CDUPRT-GDEPT is used in conjunction with the cytokine gene therapy.*
- Established that RM1-GFP/mIL12 and RM1-GFP/mIL18 secrete biologically active mIL12 and mIL18 respectively by *in vitro* assay.
- Shown that RM1-GFP/mIL12 and RM1-GFP/mIL18 inhibit tumor take rate and tumor growth after implantation either subcutaneously or in the prostate of C57BL/6 mice.
- Shown that a combination of intratumoral therapy of RM1-GFP/CDUPRT prostate tumors using pVITRO2.mIL12 + pVITRO2.mIL18 inhibits pseudometastases of RM1 cells in the lungs of C57BL/6 mice.
- Shown that a combination of intratumoral therapy of RM1-GFP/CDUPRT prostate tumors using pVITRO2.mIL12 + pVITRO2.mIL18 in mice treated with 5FC inhibits prostate tumor growth more than GDEPT alone.

REPORTABLE OUTCOMES:

- Establishment of new cell lines derived from RM1: RM1-GFP/CDUPRT; RM1-GFP/mIL12; RM1-GFP/mIL18.
- Establishment of new androgen-refractory TRAMP cell lines. (TOW prize poster) (paper in preparation)
- Determined new methodology for testing the effects of mIL12 and mIL18 and a combination of these using CTLL2 cells (paper prepared for submission to J Immunological Methods, see Appendix IV)
- Paper on local and distant bystander effects of CDUPRT-GDEPT and 5FC for PC (paper submitted to J Gene Medicine, see Appendix III).
- Poster presented for AACR conference, April, 2005, Anaheim, USA
- Poster presented for Australasian Gene Therapy Conference, April, 2005, Melbourne, Australia
- Dr Rosetta Martiniello-Wilks has been appointed as a Senior Hospital Scientist at Royal Prince Alfred Hospital to set up a GLP facility for Gene Therapy trials. She was the successful candidate for this position because she was a DOD Trainee-fellow.

CONCLUSIONS:

- At this stage of the work, we have 1 paper submitted, 2 papers in preparation and three abstracts.
- We have proof of principle that CDUPRT-GDEPT + 5FC is an effective therapy against RM1 tumor cells grown in the prostate of C57BL/6 mice. This treatment is associated with a local bystander effect, and stimulates a distant bystander effect as evidenced by inhibition of pseudo-metastasis formation in the lungs after intravenous injection of RM1 cells.
- We have clearly shown using CTLL2 cells that mIL12 and mIL18 together exhibit increased effects over either alone in increasing cellular proliferation.
- We have shown that RM1-GFP/mIL12 and RM1-GFP/mIL18 cell lines express biologically active cytokines that inhibit tumor take and tumor growth after implantation under the skin or in the prostate.
- We have shown that mIL12 and mIL18 synergize in preventing pseudo-metastases from RM1 cells in the lungs.
- We have shown that trimodal therapy, GDEPT (CDUPRT/5FC) with mIL12 and mIL18, given into the prostate, result in an increased effect against local tumor growth.

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APPENDICES:

Appendix I contains figures 1-18.

Appendix II Publications and papers in preparation

- CDUPRT paper
- CTLL2 paper in preparation
- Conference Abstracts

APPENDIX I

Figures 1-18

List of figures:

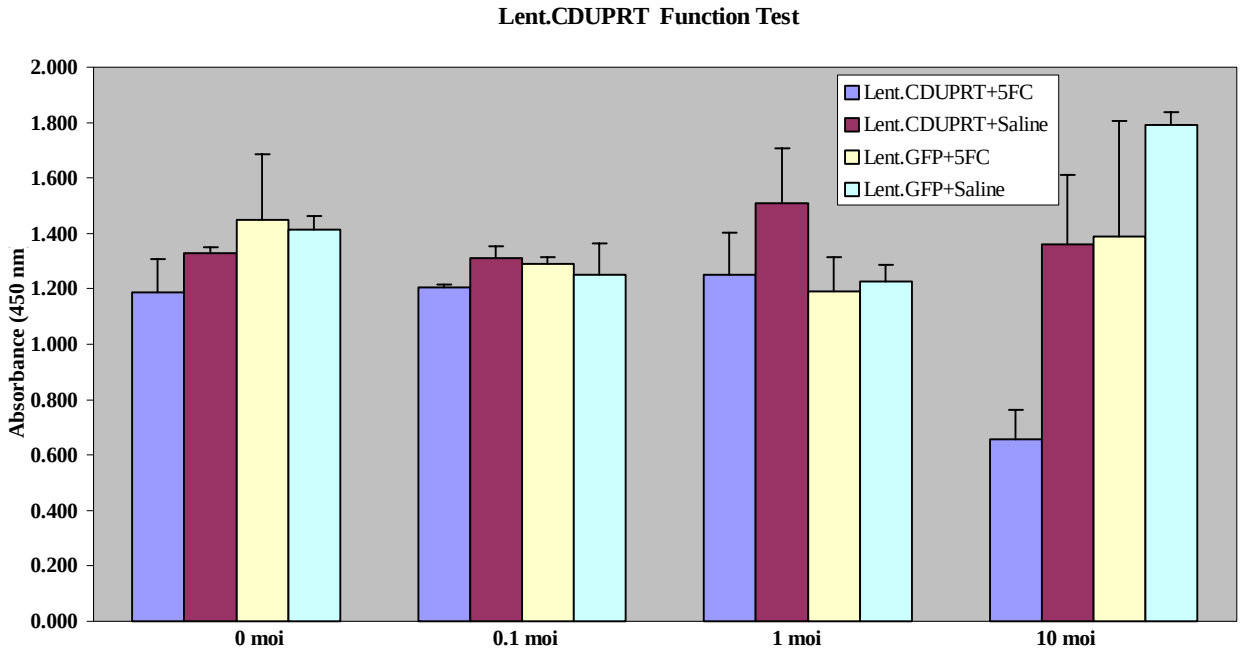
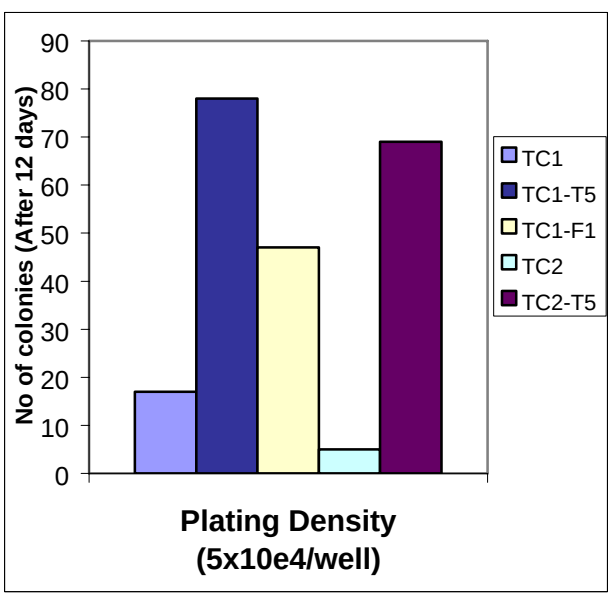


Figure 1: Functional testing of Lent.CDUPRT on RM1 cells. X-axis shows absorbance at 450nm. Lent.CDUPRT supernatants were used to transduce RM1 cells at three different doses (10, 1 and 0.1 µL/well) for 24 h and then the cells were treated with 5-fluorocytosine at 1mM concentration. 48 h post treatment, the cells were analysed for proliferation using WST1 reagent and absorbance was measured at 450nM .



<u>Figure 2: Anchorage independent growth by different TRAMP cell lines in soft agar:</u> Cell lines were plated at $5x10^4$ cells in soft agar and colony counts (>20 cells/colony) were performed microscopically at 12 days post seeding.

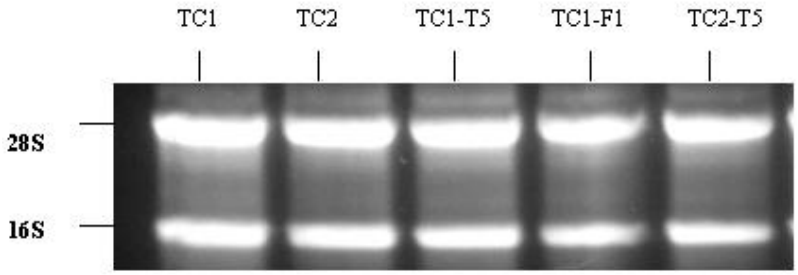
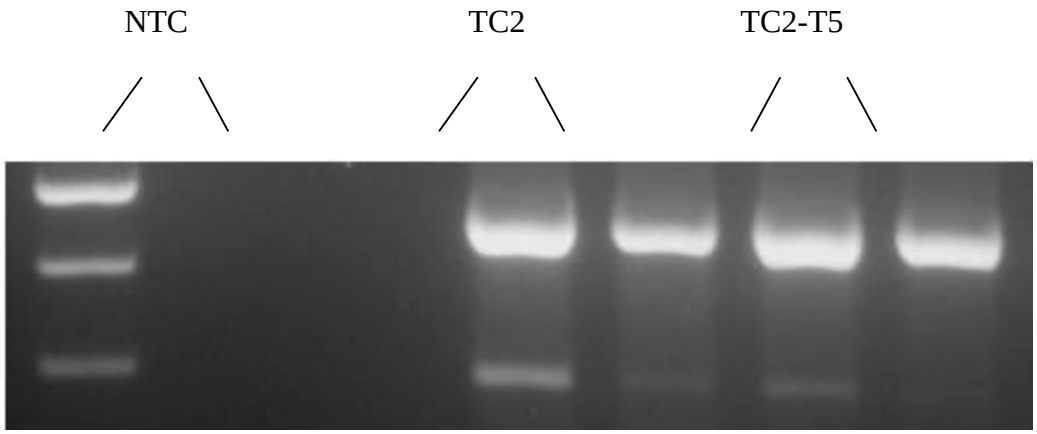


Figure 3A: Gel electrophoresis of total RNA extracted from different TRAMP cell lines.



Number of Amplification Cycles	**28**	**27**	**28**	**27**

Figure 3B: Semi quantitative multiplex RT-PCR studies to determine AR expression: Amplified fragments were resolved in 2% agarose gel & revealed by ethidium bromide staining. With the primers used, a 300 bp AR fragment was amplified. As an internal control, a 457 bp GAPDH fragment was amplified, and a no cDNA template control (NTC) served as the negative control. Samples were run at 27 or 28 amplification cycles to check the amplification rate. The derived cell line, TC2-T5, showed no amplification at 27 or 28 cycles whereas AR was amplified in the parental line, TC2, at 28 cycles but not at 27 cycles. The results indicated a significant decrease in the level of AR expression in TC2-T5 compared to TC2.

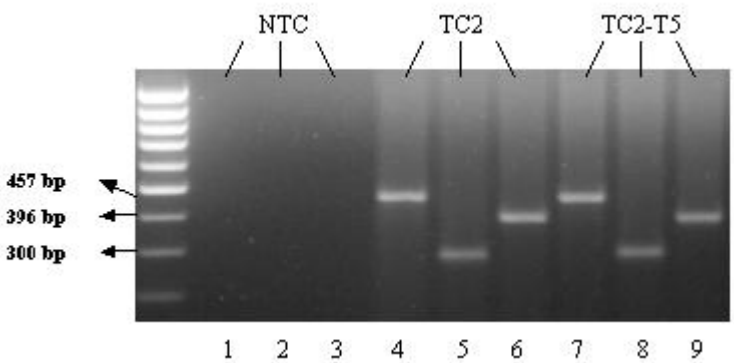


Figure 3C: Relative mRNA expression levels of AR & uPA detected by real time RT-PCR. The relative expression of AR: GAPDH was 0.7 TC2-T5 cells (Lanes 7 & 8) compared to 1.8 in the parental TC2 (Lanes 4 & 5) whilst that for uPA: GAPDH was similar in each line (Lanes 6 & 9; 1.2 and 1.3). PCR system devoid of template cDNA served as negative control (Lanes 1-3).

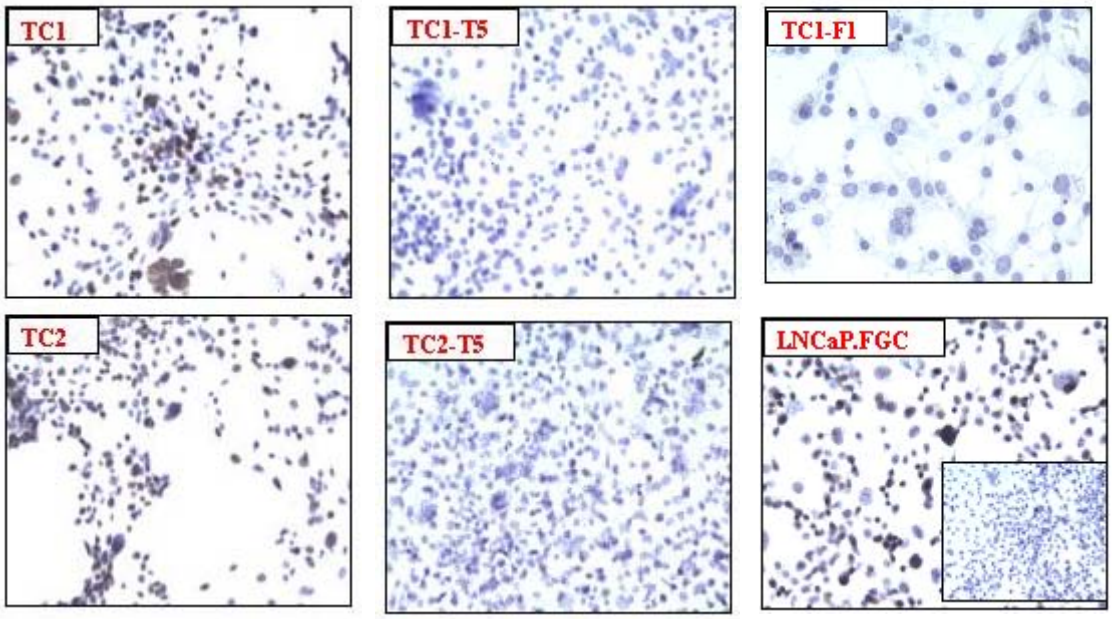


Figure 4A: Immunostaining for Androgen Receptor (AR) *in vitro*: The derived cell lines were negative for AR indicating androgen-independence whereas parental TRAMP C1 & C2 lines showed heterogenous nuclear staining indicating androgen sensitivity. LNCaP cells were used as positive control; cells treated with no primary antibody (inset) served as negative control. All slides at X 20

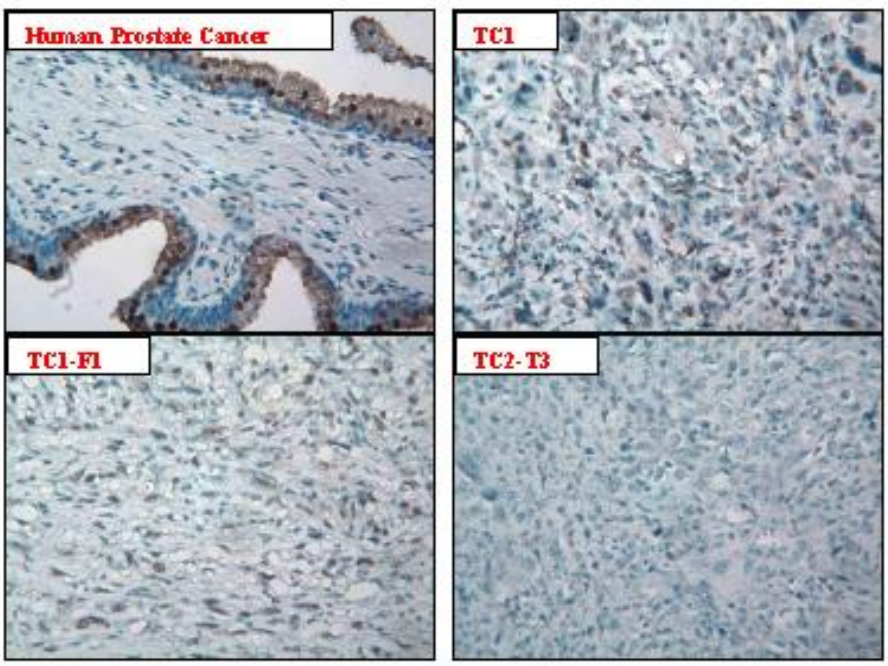


Figure 4B: Immunostaining for Androgen Receptor *in vivo* by pressure cooker antigen retrieval method (125^0 C). Parental TRAMP C1 showed weak nuclear staining; TC1-F1 showed very weak staining and TC2-T3, negative staining. Human prostate cancer tissue was used as positive control. All slides shown at X 40.

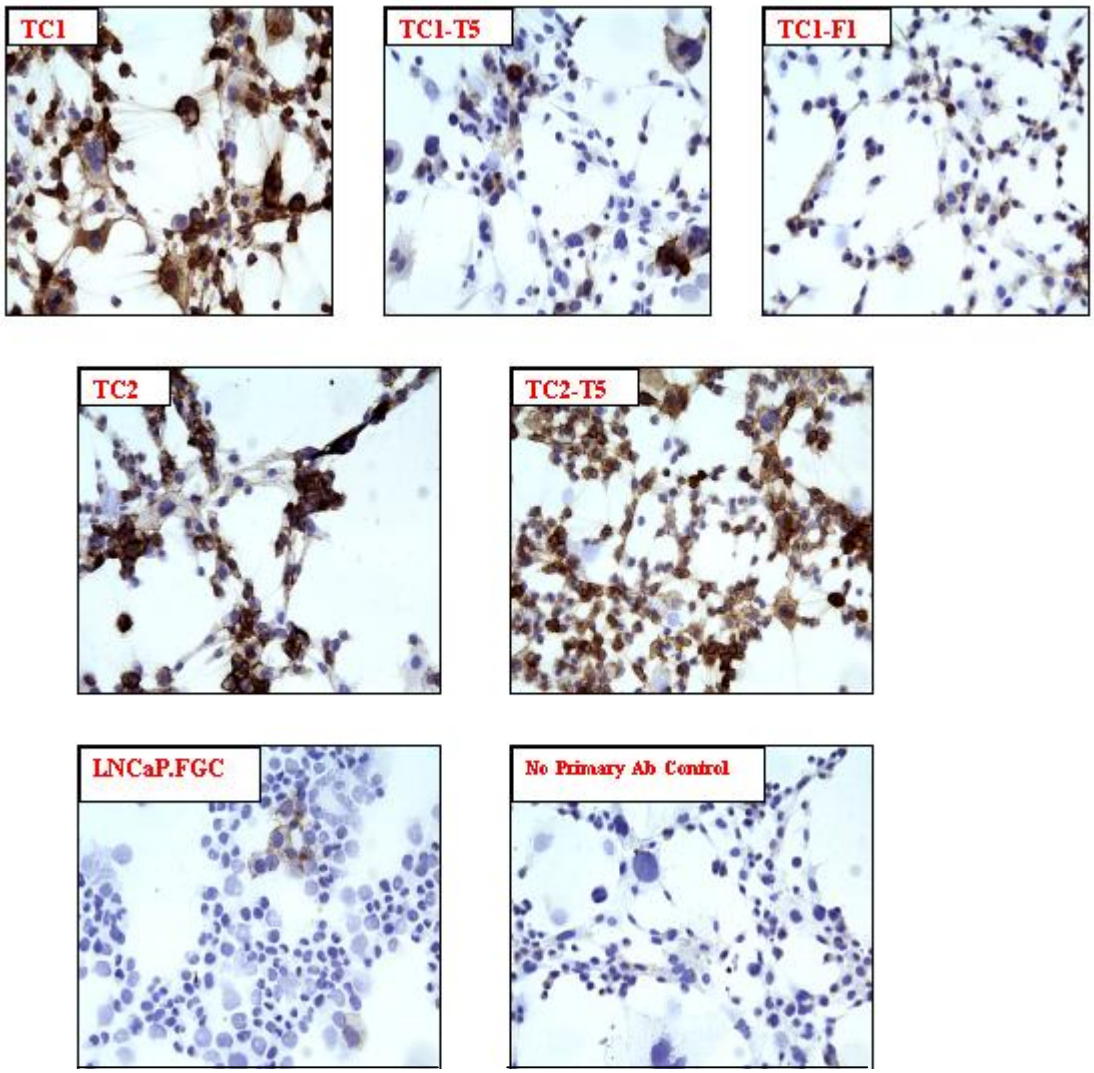


Figure 5: Immunostaining for E-Cadherin *in vitro*: The derived cell lines TC1-T5 & TC1-F1 showed very weak cytoplasmic staining compared to parental TRAMP C1 cells suggesting increased invasiveness. However, TRAMP C2 & TC2-T5 lines showed strong cytoplasmic staining. LNCaP.FGC cells with and without primary antibody served as positive and negative controls respectively. All slides shown at X 20.

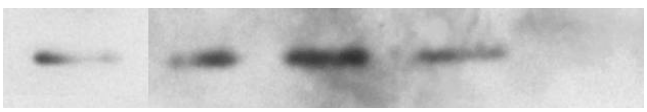


Figure 6: Western blot for mIL18 production by RM1-GFP/IL18 cells: Lane 1) recombinant IL-18 (standard). Lanes 2-4) the 3 highest cytokine expressing RM1-GFP/IL18 populations. Lane 5) RM1-GFP (control).

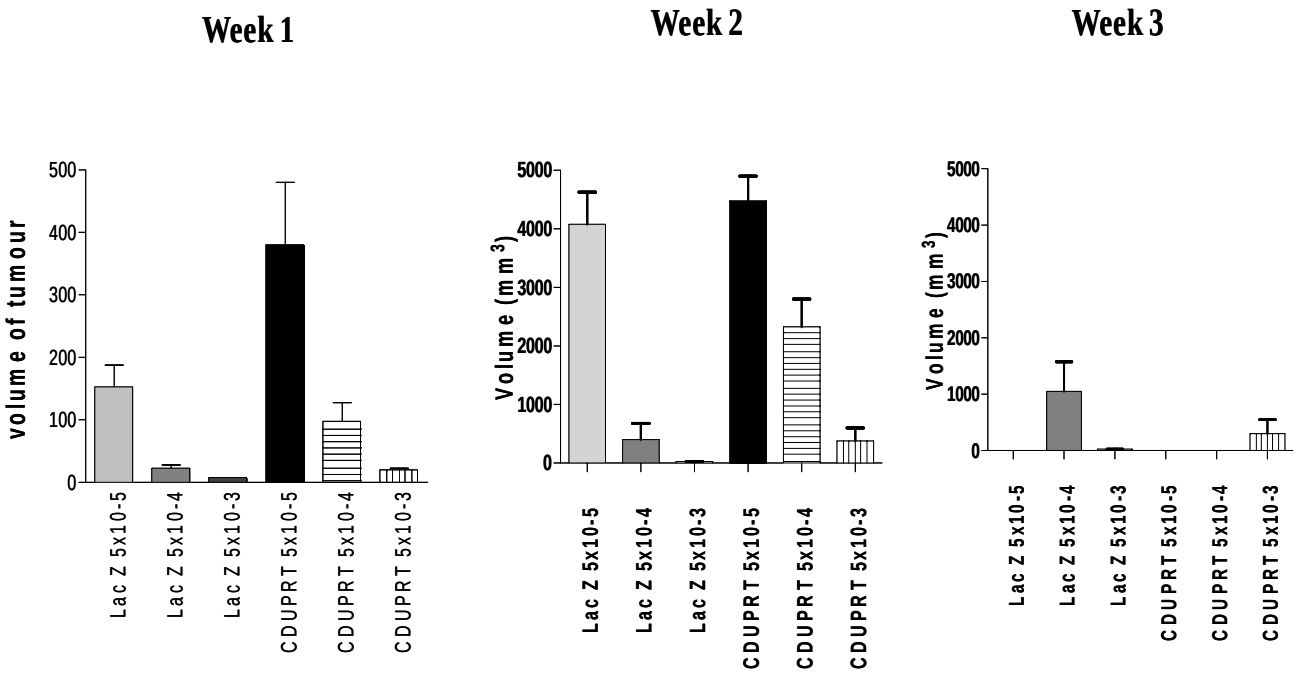


Figure 7: Growth of RM1-GFP/CDUPRT (CDUPRT) and RM1-GFP/LacZ (LacZ) tumors in the prostate of C57BL/6 mice. Mice were injected intraprostatically (Iprost) with different doses of tumor cells and the tumor growth was measured at weeks 1, 2 and 3. By week 3, mice with large tumors had to be culled (RM1-GFP/LacZ, 10^5 cells and RM1-GFP/CDUPRT 10^4 and 10^5 cells)

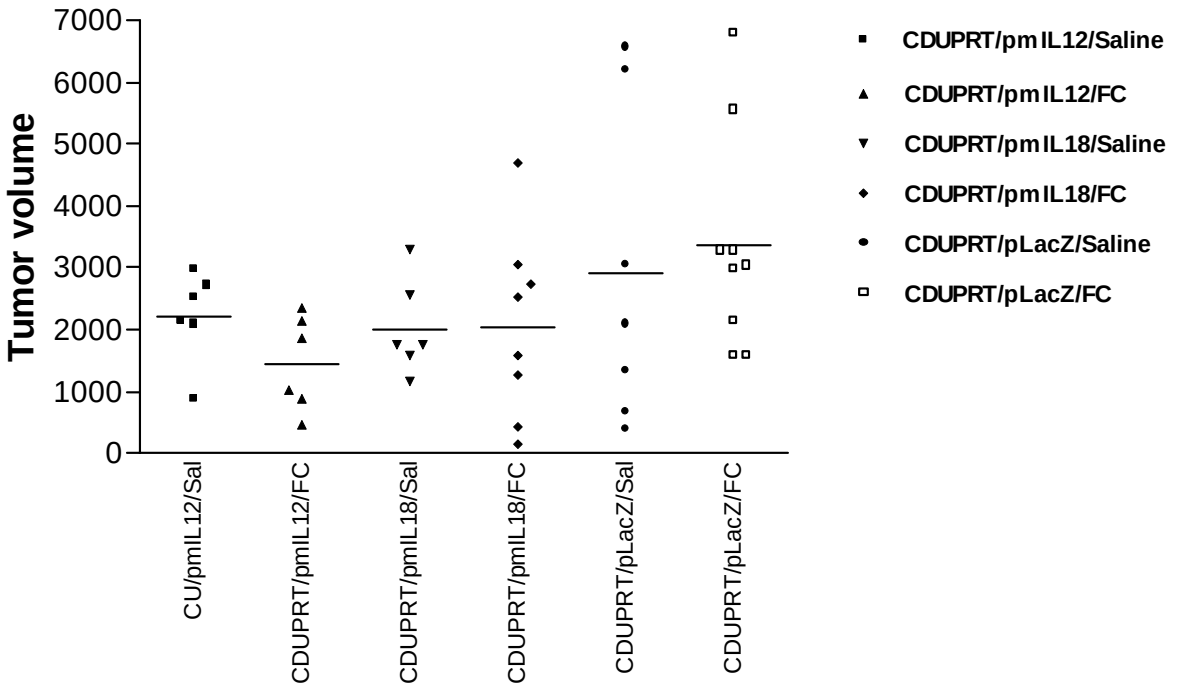


Figure 8: Efficacy of CDUPRT GDEPT in combination with pVITRO2.mIL12 or pVITRO2.mIL18 on growth of intraprostatic (Iprost) RM1 tumors in C57BL/6 mice. Mice implanted with RM1-GFP/CDUPRT (CDUPRT) tumors were injected intratumorally (i.t.)with pVITRO2.mIL12 or pVITRO2.mIL18 or pVITRO2.LacZ followed by intraperitoneal (ip)injections of 5FC or saline everyday until necroscopy. A comparison of tumor volumes between different groups is shown.

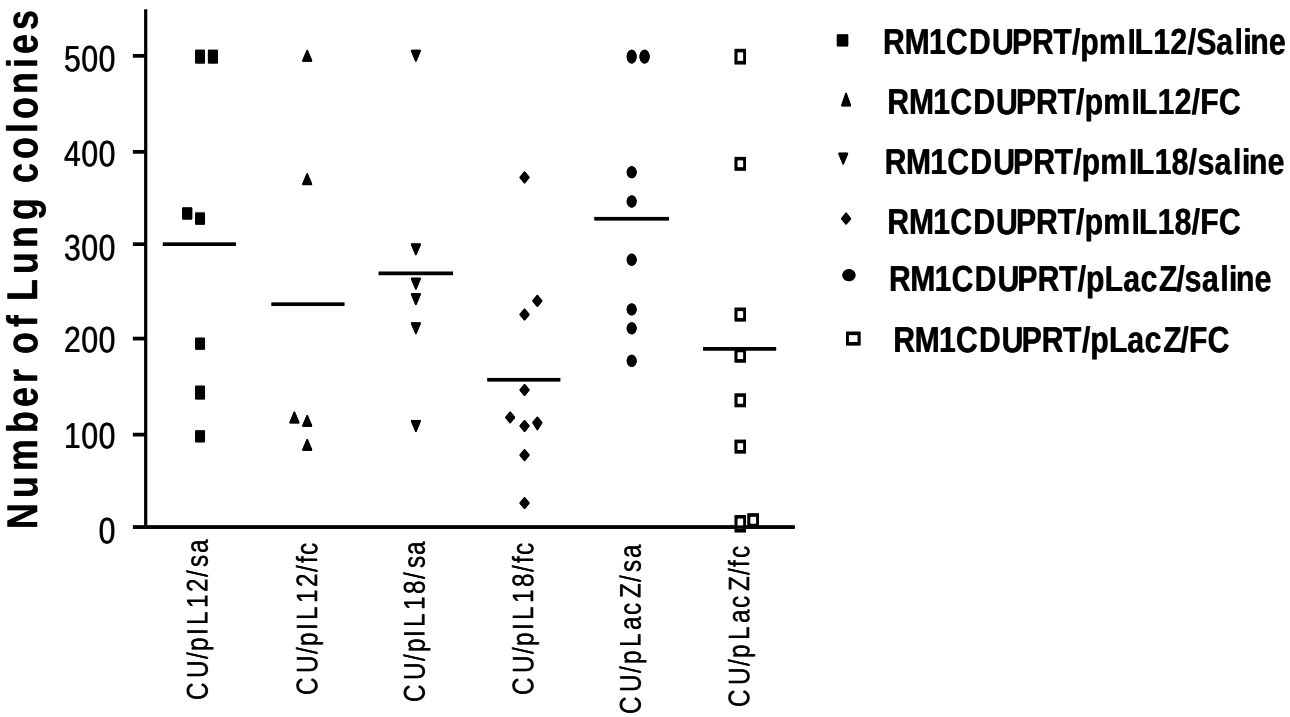


Figure 9: Distant Bystander effect: Efficacy of combination of CDUPRT GDEPT with pVITRO2.mIL12 or pVITRO2.mIL18 for treating RM1 pseudometastases in lungs of C57BL/6 mice. Mice implanted with RM1-GFP/CDUPRT (CU) tumors were injected intratumorally with pVITRO2.mIL12 (pIL12), pVITRO2.mIL18 (pIL18) or pVITRO2.LacZ (pLacZ). On day 6. mice were injected intravenously with parental RM1 cells to establish pseudometastases in lungs and 5fluorocytosine (fc) or saline (sa) were given intraperitoneally daily until necroscopy (day 16) . At necroscopy, lungs were fixed in Bouin's reagent and colony count was done under the dissecting microscope. Lung colony counts of different treatment groups are shown.

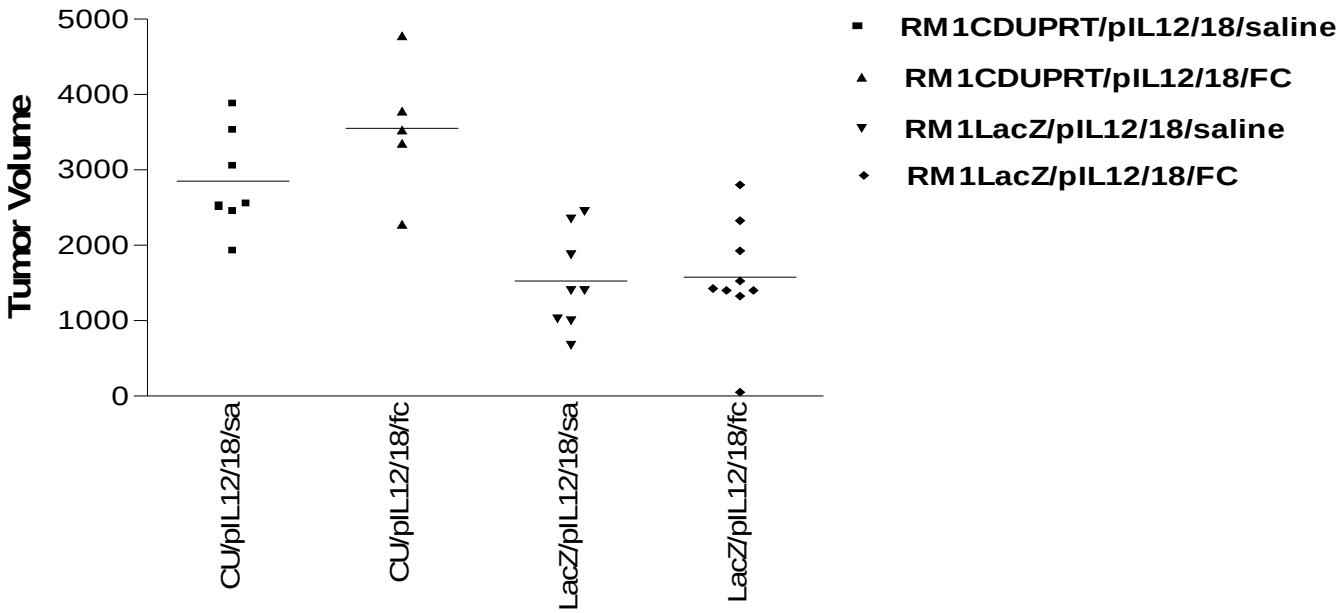


Figure 10: Effects of CDUPRT GDEPT in combination with pVITRO2.mIL12 and pVITRO2.mIL18 on growth of intraprostatic RM1 tumors in C57BL/6 mice. Mice implanted with RM1-GFP/CDUPRT (CU) tumors or with RM1-GFP/LacZ (LacZ) were injected intratumorally with pVITRO2.mIL12 and pVITRO2.mIL18 (pIL12/18) followed by 5-fluorocytosine (fc) or saline (sa) given intraperitoneally daily until necroscopy (day 16).

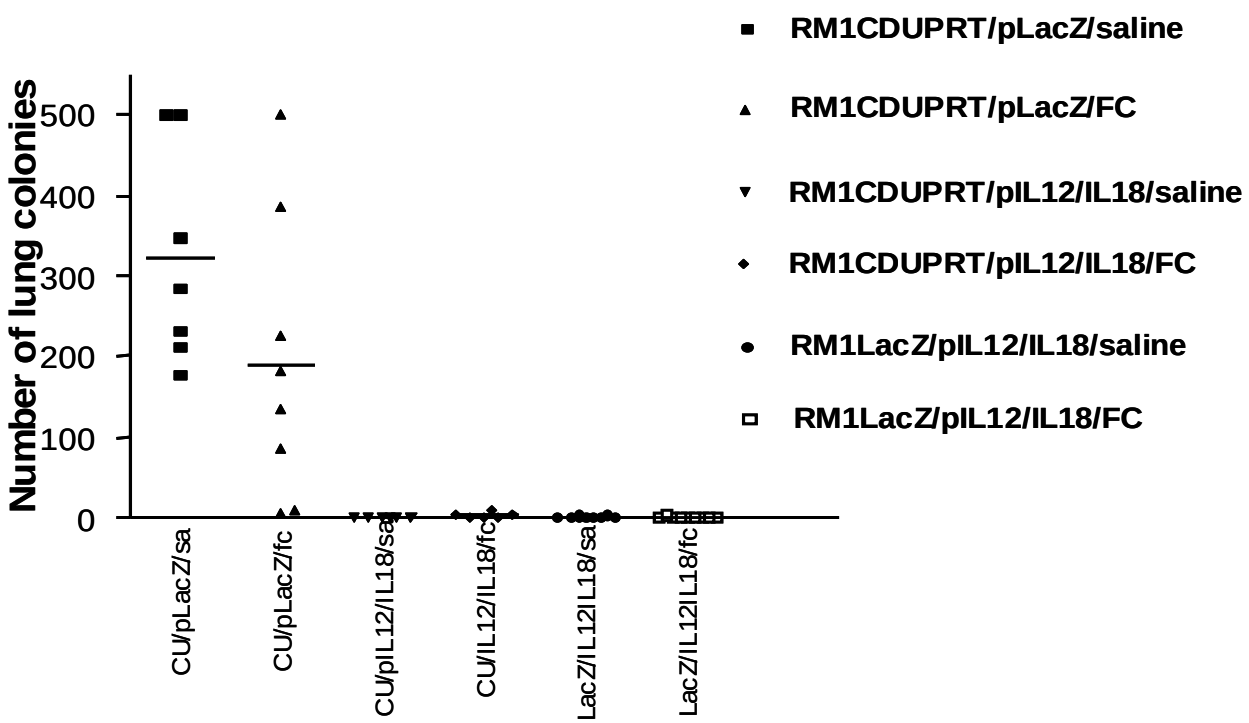


Figure 11A: Distant bystander effect of the trimodal therapy: Efficacy of combination of CDUPRT GDEPT with pVITRO2.mIL12 and pVITRO2.mIL18 on RM1 pseudometastases in C57BL/6 mice. Mice implanted with RM1-GFP/CDUPRT (CU) or RM1-GFP/LacZ (LacZ) tumors were injected intratumorally with pVITRO2.mIL12 and pVITRO2.mIL18 (pIL12/IL18) or with pVITRO2.LacZ (pLacZ). On day 6. mice were injected intravenously with parental RM1 cells to establish pseudometastases in lungs and 5-fluorocytosine (fc) or saline (sa) were given ip daily until necroscopy. Lungs were then fixed in Bouin's reagent and colony counts were done. Lung colony counts in different treatment groups are shown. RM1LacZ cells and pVITRO2.LacZ plasmid were used as controls.

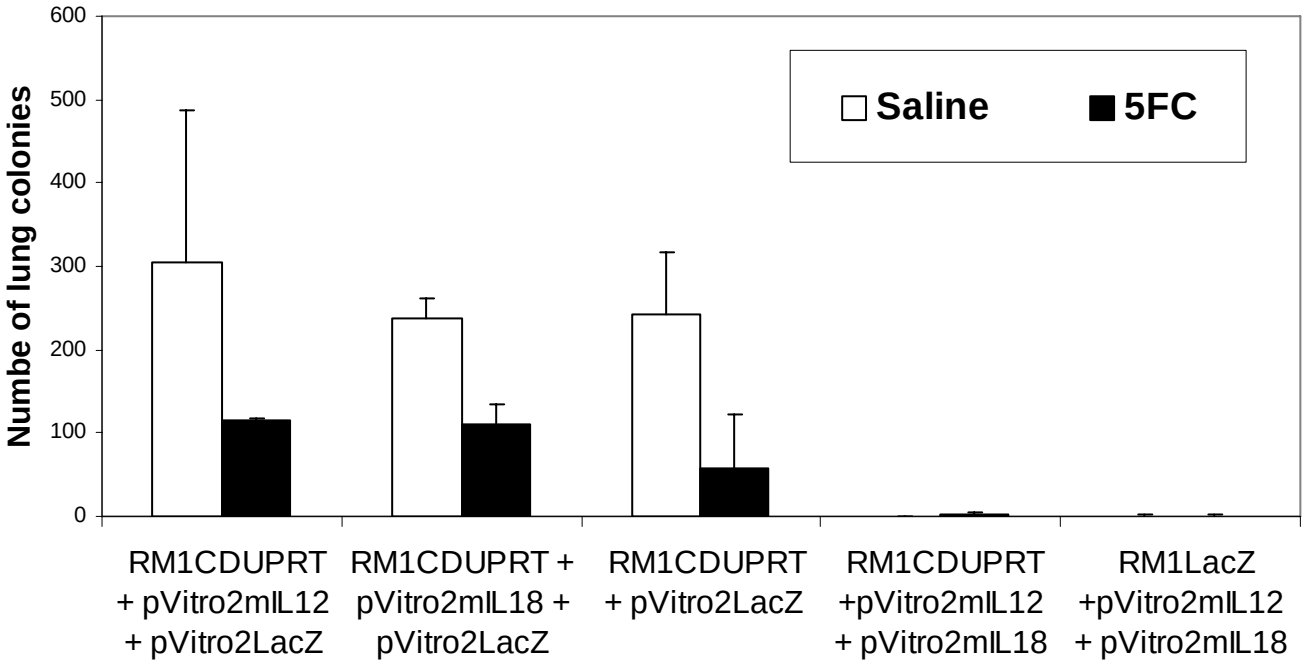


Figure 11B: **Distant Bystander effect: Efficacy of combination of CDUPRT GDEPT with pVITRO2.mIL12 or pVITRO2.mIL18 or both for treating RM1 pseudometastases in lungs of C57BL/6 mice.** Mice implanted with RM1-GFP/CDUPRT (RM1CDUPRT) or RM1-GFP/LAcZ (RM1LacZ) tumors were injected intratumorally with pVITRO2.mIL12 + pVITRO2.LacZ, pVITRO2.mIL18 + pVITRO2.LacZ or pVITRO2.mIL12 + pVITRO2.mIL18. On day 6. mice were injected intravenously with parental RM1 cells to establish pseudometastases in lungs and 5-fluorocytosine (5FC) or saline were given intraperitoneally daily until necroscopy (day 16) . At necroscopy, lungs were fixed in Bouin's reagent and colony counts were done under the dissecting microscope. Lung colony counts of different treatment groups are shown.

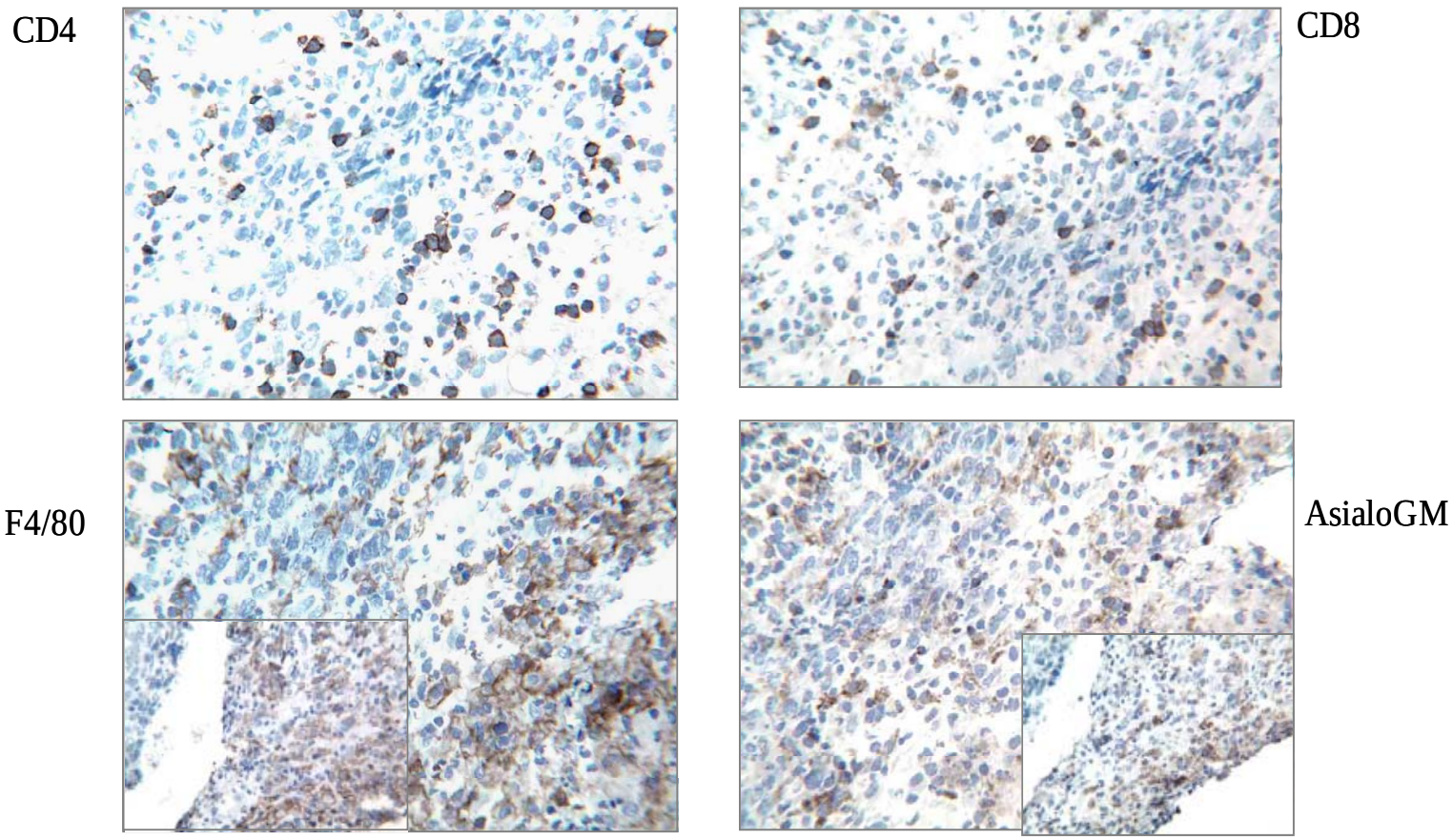
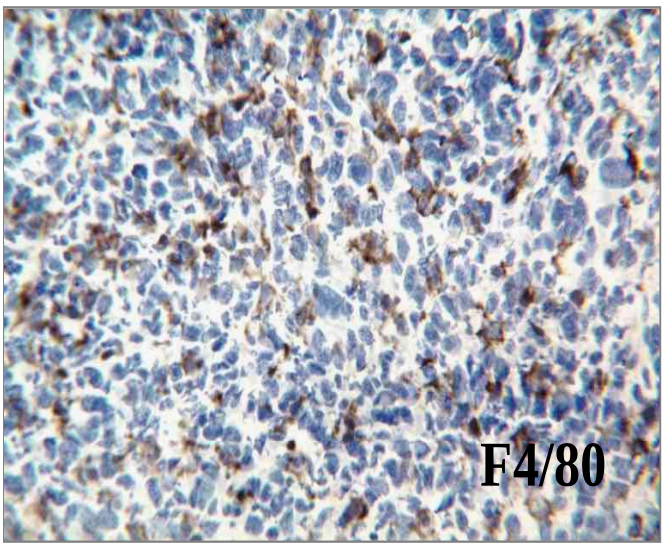
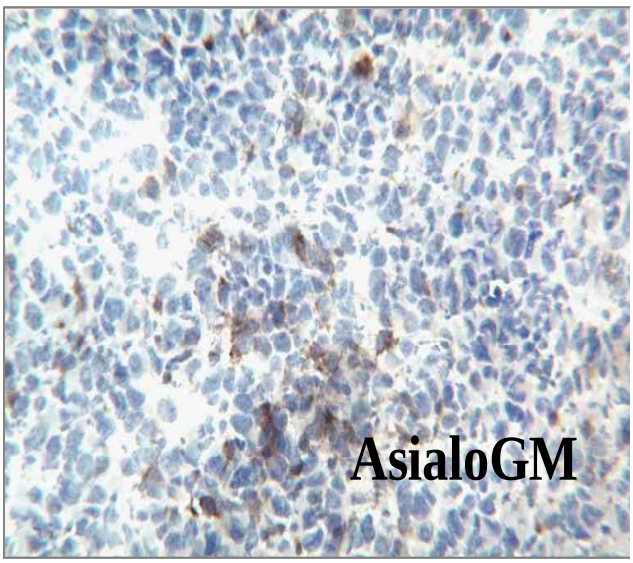


Figure 12: Infiltration of RM1LacZ prostates treated with pVITRO2.mIL12 plus pVITRO2.mIL18 (i.t.) and given 5FC daily ip until euthanasia. CD4+ and CD8+ T cells, F4/80+ macrophages and AsialoGM+ cells were present. An additional tumour outgrowth was rich in macrophages, but not NK cells (inserts)

Mouse #103.2





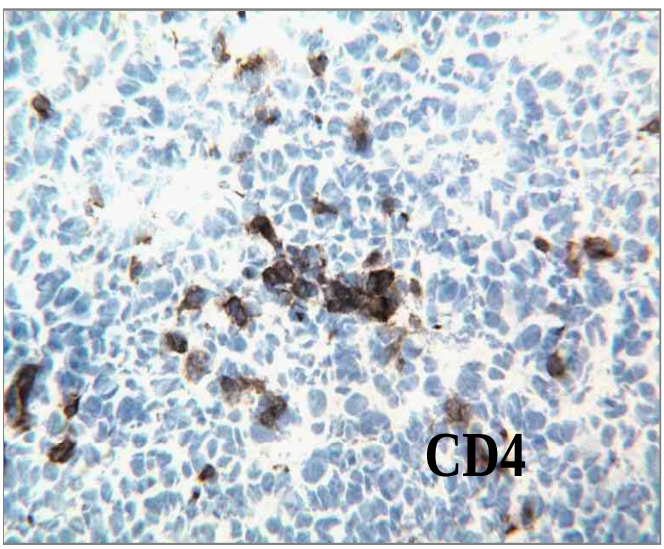
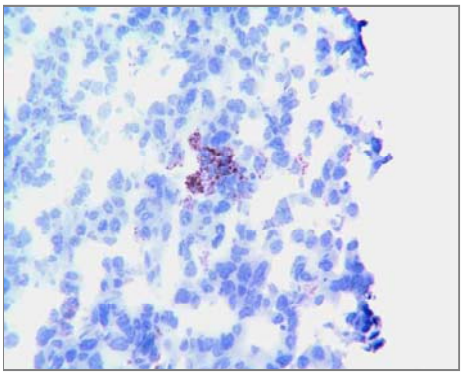
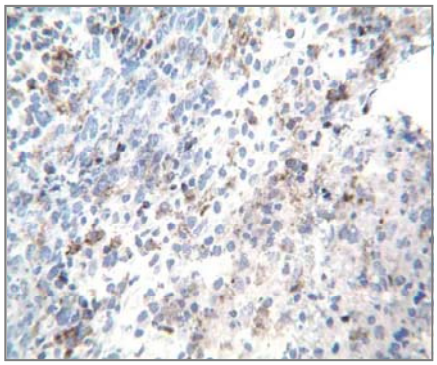
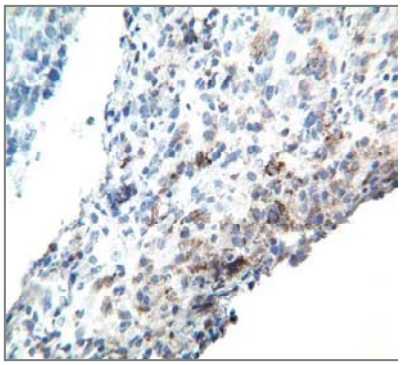


Figure 13: Immune cells in RM1LacZ iprost tumors treated with pIL12/pIL18 (intratumorally) then saline daily intraperitoneally until euthanasia (day 16). Tumors were infiltrated with macrophages (F4/80), NK (AsialoGM) and CD4 T cells.

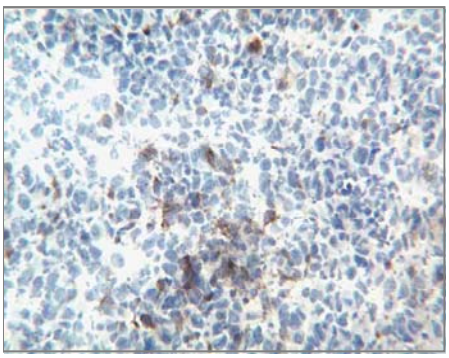
Asialo GM





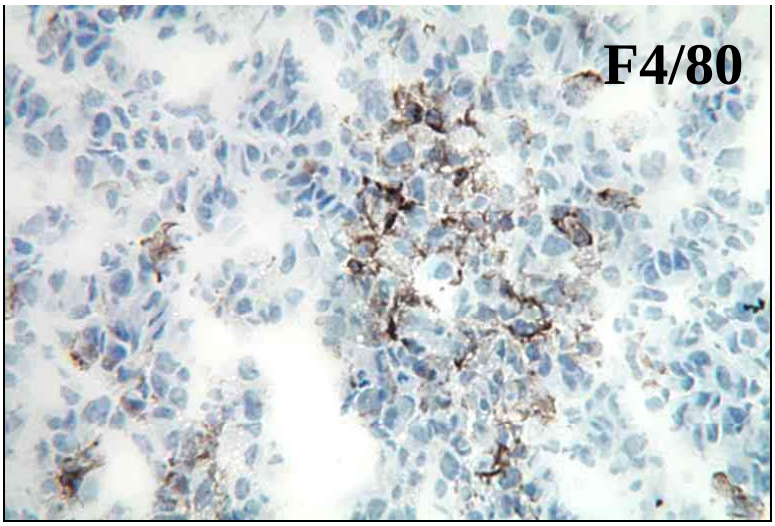


RM1-GFP/CDUPRT treated with pIL12/pIL18, then 5FC daily until euthanasia (day 16)



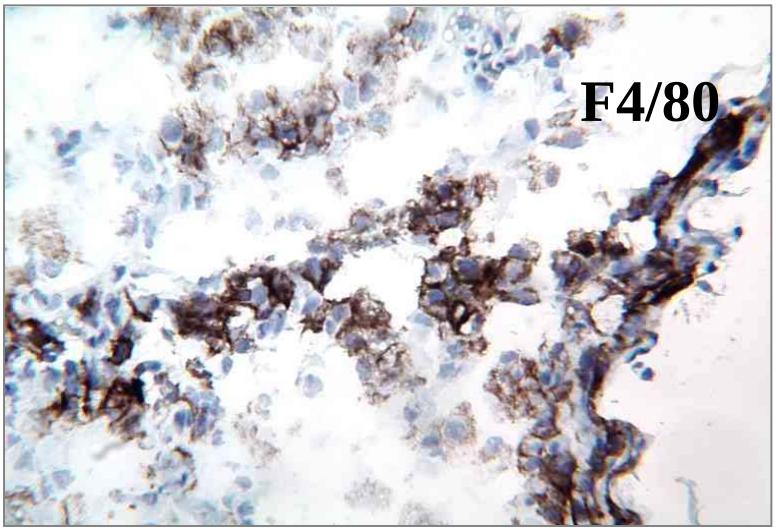
RM1-GFP/LacZ tumors given pIL12/IL18/saline

Figure 14: Presence of Asialo GM+ cells in RM1 prostate cancers: Asialo GM+ cells that include NK cells were present in RM1 prostate cancers harvested after various treatments as shown.



Mouse #96.2

RM1-GFP/CDUPRT tumor treated with pIL12/IL18 and 5FC



Mouse #96.3

RM1-GFP/CDUPRT tumor treated with pIL12/IL18 and saline

Figure 15: Comparison of tumor infiltrates in the presence and absence of GDEPT: F4/80 cells were present in higher numbers in RM1-GFP/CDUPRT prostate tumors treated with both pmIL12 + pmIL18 in mice treated with saline compared with those given 5 fluorocytosine (5FC), suggesting an immunosuppressive effect of 5-Fluorouridine generated by GDEPT.

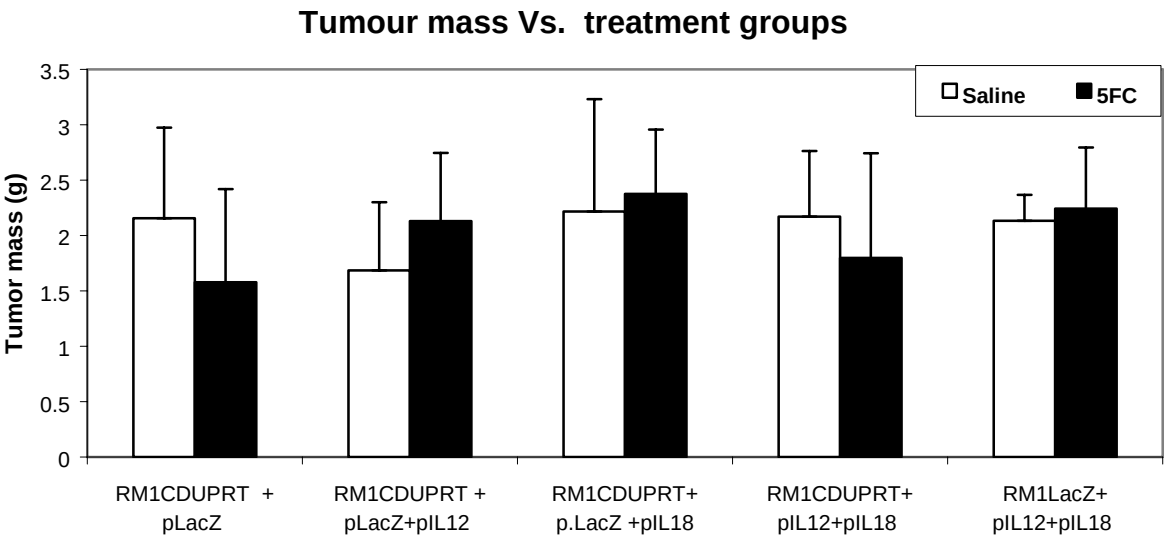


Figure 16: Efficacy of CDUPRT GDEPT in combination with pVITRO2.mIL12 and/or pVITRO2.mIL18 on growth of intraprostatic (Iprost) RM1 tumors in C57BL/6 mice. Mice implanted with RM1CDUPRT tumors were injected intratumorally (i.t.)with pVITRO2.mIL12 and/or pVITRO2.mIL18 or pVITRO2.LacZ followed by intraperitoneal (ip)injections of 5FC or saline everyday until necroscopy. A comparison of tumor volumes between different groups is shown.

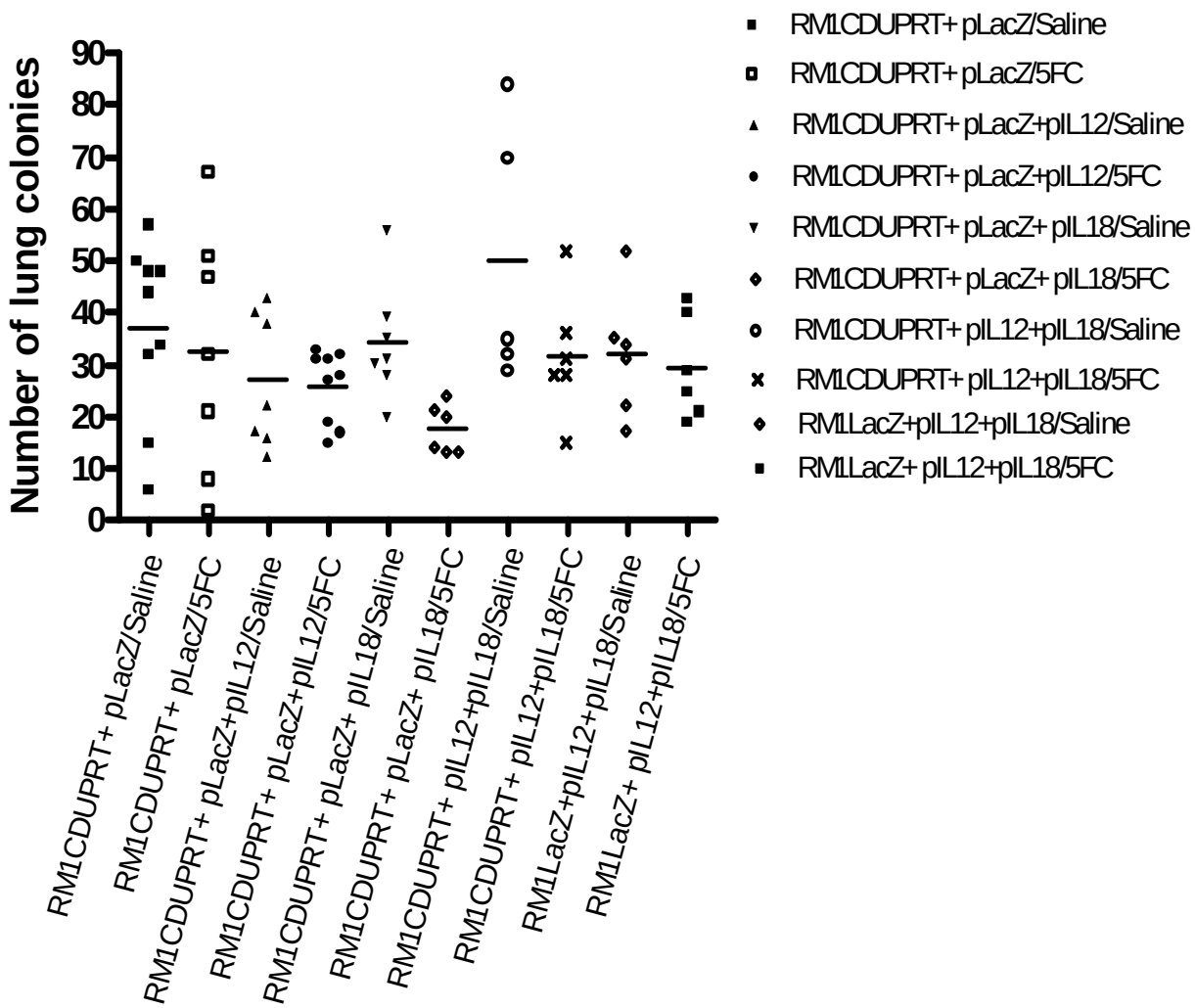
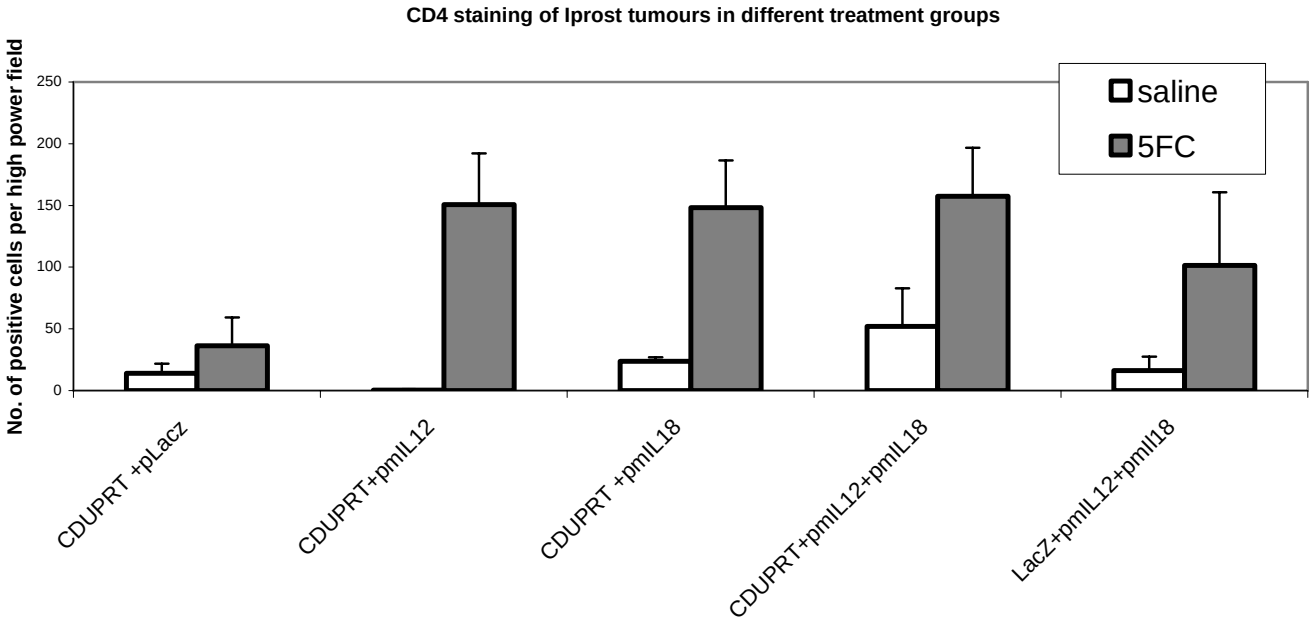


Figure 17: Distant Bystander effect of combination therapy: Efficacy of combination of CDUPRT GDEPT with pVITRO2.mIL12 and pVITRO2.mIL18 on RM1 pseudometastases in C57BL/6 mice. Mice implanted with RM1CDUPRT or RM1LacZ tumors were injected intratumorally with pVITRO2.mIL12 + pVITRO2.LacZ, pVITRO2.mIL18 + pVITRO2.LacZ or pVITRO2.mIL12 + pVITRO2.mIL18. . On day 7. mice were injected intravenously with parental RM1 cells to establish pseudometastases in lungs and 5-fluorocytosine (5FC) or saline were given intraperitoneally daily until necroscopy. At necroscopy, lungs were fixed in Bouin's reagent and colony counts were done under the dissecting microscope. Lung colony counts of different treatment groups are shown.



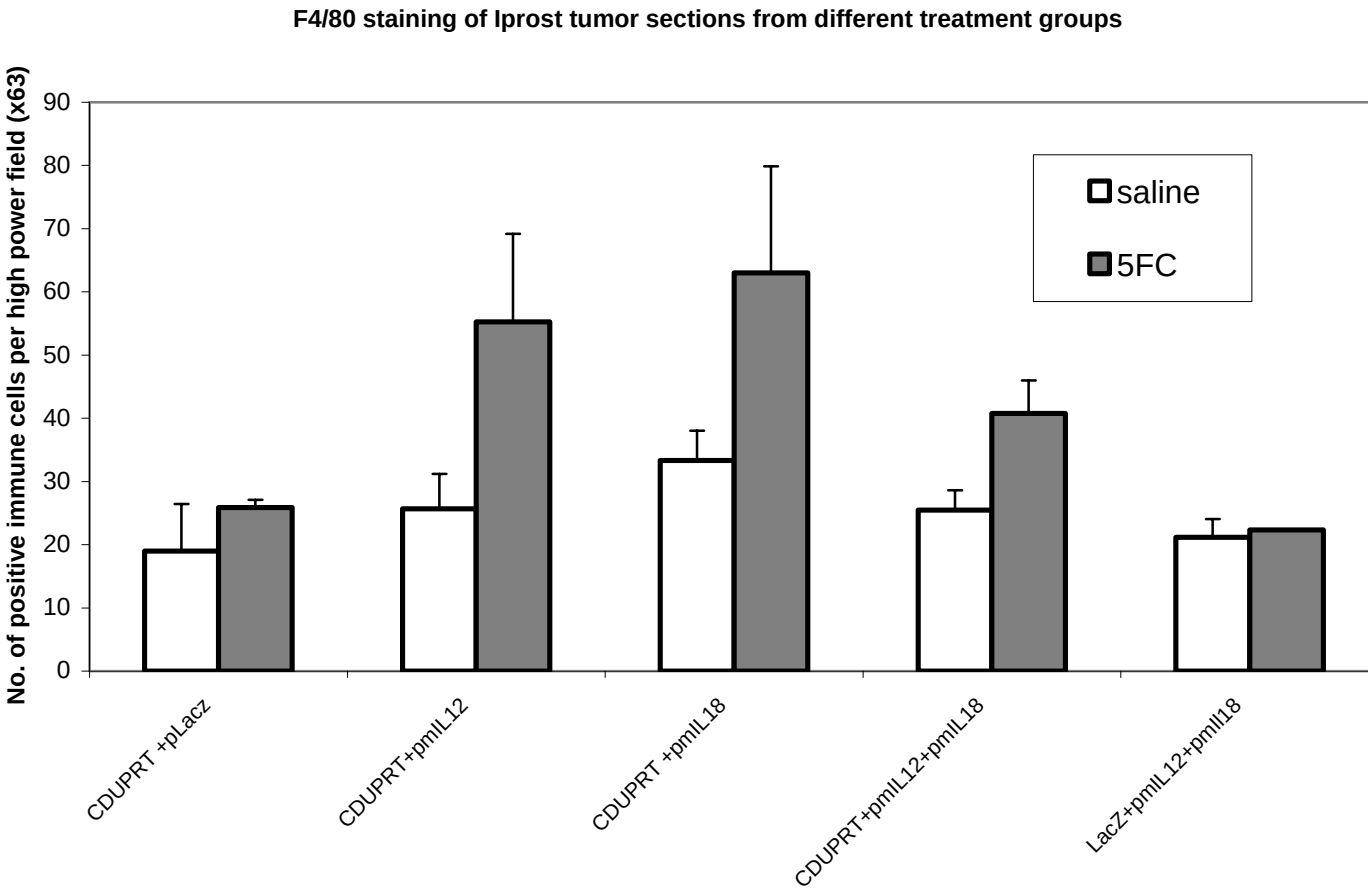


Figure 18: Intratumoral infiltrating immune cells following CDUPRT-GDEPT/Cytokine treatment. Panel A shows CD4 staining of Iprost tumors in different treatment groups and panel B shows F4/80 staining of Iprost tumor sections from different treatment groups.

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Title: Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against prostate-cancer in C57BL/6 mice**.**

Article Type: Regular Article

Section/Category:

Keywords: GDEPT; CDUPRT gene; RM1 model; 5 fluorocytosine; local bystander effect

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Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against RM1 prostate-cancer in mice.

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Running Title: CDUPRT-GDEPT using the mouse RM1 prostate cancer model

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Abbreviations Used: AR, Androgen refractory; AS, Androgen sensitive; PC, Cancer of the prostate; FBS, Fetal bovine serum; GDEPT, Gene directed enzyme prodrug therapy; HSVtk, Thymidine kinase gene from Herpes Simplex Virus; PNP, purine nucleoside phosphorylase. HRPC, Hormone refractory prostate cancer.CD, cytosine deaminase; UPRT, uracil phosphoribosyl transferase; CDUPRT, cytosine deaminase in combination with uracil phosphoribosyl transferase. 5FC, 5-fluorocytosine; 5FU, 5-fluorouracil; GFP, Green Fluorescent protein; HPLC, High performance liquid chromatography; 5FdUMP, 5-fluoro-2'-deoxyuridine 5'monophosphate; 5FUTP, 5-fluorouridine 5'-triphosphate; 5FUMP, 5-fluorouridinemono-phosphate; ICGJ, Intercellular gap junctions; NK cells, Natural killer cells.

ABSTRACT
We aimed to evaluate the efficacy of gene–directed enzyme prodrug therapy (GDEPT) using cytosine deaminase in combination with uracil phosphoribosyl transferase (CDUPRT) against intra-prostatic mouse androgen-refractory prostate (RM1) tumors in immunocompetent mice. The product of the fusion gene, CDUPRT, converts the prodrug, 5-fluorocytosine (5FC) to 5-fluorouracil (5FU) and other cytotoxic metabolites that kill both CDUPRT-expressing and surrounding cells, via a 'bystander effect'. RM1 cells were stably transfected with plasmids containing green fluorescence protein (GFP)/CDUPRT, GFP or GFP/LacZ genes. CDUPRT expression in RM1-GFP/CDUPRT cells or tumors was confirmed by enzymic conversion of 5FC to 5FU, using HPLC. Treatment of mice bearing intra-prostatic RM1-GFP/CDUPRT tumors with 5FC resulted in complete regression of the tumors. A 'local bystander effect' was seen, even though only 20% of the cells expressed CDUPRT. More importantly a significant reduction in pseudo-metastases of RM1 cells in lungs indicated a 'distant bystander effect'. Immunohistochemical evaluation of the treated tumours showed increased necrosis and apoptosis, with decreased tumor vascularity. There was also a significant increase in tumour-infiltration by macrophages, $CD4^+$ T and natural killer cells. We conclude that CDUPRT-GDEPT significantly suppressed the aggressive growth of RM1 prostate-tumors and lung pseudo-metastases via immune mechanisms involving necrosis and apoptosis.

KEYWORDS: GDEPT; CDUPRT gene; RM1 model; 5 fluorocytosine; local bystander effect

INTRODUCTION

Of several GDEPT systems under investigation for PC (1), we are evaluating a fusion gene constructed by combining cytosine deaminase (CD) with uracil phosphoribosyltransferase (UPRT). Cytosine Deaminase is an enzyme of bacterial or fungal origin that converts the non-toxic pro-drug, 5 fluorocytosine (5FC) to 5-fluorouracil (5FU). 5FU is further modified by cellular enzymes to pyrimidine antimetabolites, 5-fluoro-2'-deoxyuridine 5'monophosphate (5FdUMP) and 5-fluorouridine 5'-triphosphate (5FUTP) that inhibit DNA and RNA synthesis. Thus, 5FU should be effective in killing both dividing and non-dividing cells, an important factor, given the low percentage (2%) of dividing cells in PC (2). Despite the success of 5FU-chemotherapy for gastrointestinal, head and neck malignancies, it has shown a poor therapeutic index for other cancers primarily because some tumor cells develop immunity to 5FU toxicity. A major factor responsible is the low efficiency of conversion of 5FU into its toxic metabolites; the rate-limiting step is the formation of an intermediary metabolite, 5-fluorouridinemono-phosphate (5FUMP) involving a series of enzyme-catalyzed reactions (3). This is circumvented by the ability of UPRT to convert 5FU directly to 5FUMP leading to more efficient production of toxic metabolites (4) and hence increased sensitization (up to 15 fold) of the UPRT-transduced cells to 5FU (5). Use of UPRT in conjunction with CD sensitizes cancer cells to low doses of 5FU and 5FC (3,6,7) and results in greater anti-tumor efficacy compared with CD-GDEPT alone (7,8). Only limited studies have investigated the effects of CDUPRT GDEPT *in vivo* against PC.
Given that a 100% gene transfer efficiency with currently used gene delivery technology is unattainable *in vivo*, an important advantage of GDEPT is derived from the local bystander effect engendered; comprehensive cell killing is achieved without the need to express the gene in all cells (9,10). The local bystander effects associated with 5FC/5FU-based GDEPT is due to the ability of 5FU to freely diffuse between cells (7,11). However, the metabolites of 5FU can only

move through gap junctions (12) and therefore the extent of the bystander effects relies on the incidence of gap junctions in the cells. While the combination CDUPRT-GDEPT clearly results in a more efficient conversion of 5FU to its toxic metabolites, it may also lead to depletion of the cytoplasmic pool of freely diffusible 5FU that could compromise the bystander effect. Clearly, this needs to be investigated individually for different cell types. Although *in vitro* studies have shown that the local bystander effect associated with CDUPRT-GDEPT varies depending upon the cancer cell properties (7,11,13), none have evaluated the bystander effects against PC *in vivo*. Hence, in this study we sought to assess the local bystander effects of CDUPRT *in vitro* and *in vivo* against murine PC. Some GDEPTs including CD- and UPRT- lead to tumor cell killing in the remote locations, culminating in a *distant bystander effect* (1,14,15), which is mediated via treatment specific anti-tumor immune responses. To date there are no reports of any preclinical studies evaluating the distant bystander effects generated using the fusion gene, CDUPRT, so, we assessed its ability to produce a similar '*distant bystander effect'*.
An immunocompetent mouse model of syngeneic PC was used to examine the ability of locally given GDEPT to exert local and distant bystander effects. An androgen-refractory PC cell line (RM1) (16) was implanted into the prostate or tail vein of C57BL/6 mice leading to tumor formation and experimental lung metastasis, respectively, in an aggressive and reproducible manner (17).

RESULTS

1. Efficacy of CDUPRT-GDEPT *in vivo*

Optimization of 5FC dose: An important feature of CDUPRT GDEPT is that both 5FC and 5FU (FDA approved drugs) can be used as prodrugs for this system. We chose 5FC as it is efficiently metabolized by the fusion gene and is non-toxic at the therapeutic doses used. We first determined the maximum non-toxic dose of prodrug usable in our model. C57BL/6 mice could tolerate 5FC at doses up to 500 mg/kg/mouse, given intraperitoneally (ip) every day for 14 days without systemic toxicity as shown by examination of Hematoxylin and Eosin (H&E) stained major organs (Fig.1A) and by serum analysis for biochemical markers of kidney and liver function (Urea Creatinine, ALP, ALT and AST (Fig.1B). Neither serum markers, nor histology differed between 5FC-injected versus control mice, suggesting that there was no detectable toxicity from 5FC even at the highest dose tested. There was no loss of weight or condition in the treated mice.
Generation of stably transformed RM1 cell lines expressing GFP, GFP CDUPRT or GFPLacZ: RM1 cells were stably transformed to express GFPCDUPRT, GFP or GFPLacZ (RM1GFP, -CDUPRT or -LacZ) and gene expression of the transgenes was established. For CDUPRT, *in vitro* RM1-CDUPRT cultures and tumors harvested after subcutaneous (sc) and intraprostatic (Iprost) growth in C57BL/6 mice were shown by HPLC to have the capacity to convert 5FC to 5FU, confirming CDUPRT expression and importantly, there was no loss of activity *in vivo* over the duration of the experiments (data not shown). GFP expression in all cell lines was confirmed by UV microscopy and flow cytometry.
Evaluation of RM1 cells for assessment of CDUPRT GDEPT: Given that some cells are resistant to 5FU therapy, toxicity of 5FU to RM1 cells was assessed *in vitro* to evaluate the suitability of our model for evaluating *CDUPRT GDEPT*. Cultured RM1 cells were subjected to different doses of 5FU for 1 week (data not shown) and then assessed for viability by cell counting via trypan blue exclusion. There was almost complete eradication of cells at dose > 10μg/mL (77μM, 85% cell death) at 72 h post treatment. Cytotoxic effects were seen (~57% cell death compared with untreated controls) even at 1μg/mL (7.7μM) by day 7, which was lower than that for the human PC DU145 cells (17μM) (13) but higher than the acceptable therapeutic range of 5FU in humans (1.9 ± 0.3μM). Although, doses lower than 7.7μM were not tested, these data suggested that RM1 cells are moderately sensitive to 5FU toxicity. When tested for 5FC sensitivity, control

RM1-LacZ cells were resistant up to the highest dose tested (100μg/ml) but RM1-CDUPRT cells were susceptible to cell killing even at 3μg/ml showing ~34 fold enhancement of 5FC sensitisation of RM1–CDUPRT cells (data not shown). This clearly established the suitability of our model for assessing CDUPRT-GDEPT.

GDEPT using RM1CDUPRT/5FC: To establish that RM1-CDUPRT cells could effect GDEPT *in vivo* in the presence of 5FC, mice were implanted Iprost with RM1CDUPRT or RM1-LacZ tumors and injected ip with 5FC or saline. GDEPT in the RM1-CDUPRT/5FC group was very effective with almost complete absence of growth in the prostate compared to mice in control groups (RM1-LacZ/5FC, RM1-CDUPRT/saline) (Fig.2A)(P=0.009). Histology of RM1-CDUPRT tumors from mice treated with saline (6 mice) or 5FC (10 mice) showed a highly vascularized and viable tumor (Fig 2B) in the former, whereas treatment with 5FC resulted in necrosis (Fig 2C), with loss of prostate tissue architecture (Fig 2C, inset). All **t**umors from CDUPRT/saline mice showed >80% viability, with <10% tumor necrosis or haemorrhagic necrosis (necrosis due to disruption of vasculature). In contrast, mice given 5FC had no tumors (in 6 cases), or <10% viable tumor (4 cases) with >30% necrosis, and >60% haemorrhagic necrosis in the latter (Table 1). Other tissues examined histologically (kidney, lung, spleen, liver), showed no abnormalities (data not shown), indicating the tumor-specific nature of the cytotoxic effects of the treatment with no apparent systemic toxicity. Finally, HPLC analysis of sera from mice from RM1-CDUPRT/5FC and RM1-LacZ/5FC groups showed no detectable levels of 5FC and 5FU in either (data not shown).

2. Local bystander effect on RM1 growth

Once the efficacy of the CDUPRT GDEPT was established, any local bystander effects resulting from the treatment were assessed *in vitro* and *in vivo*.

When examined *in vitro*, medium collected from RM1-CDUPRT/5FC but not from RM1-LacZ/5FC cells prevented the growth of RM1 cells ($p<0.0001$) indicating a CDUPRT-specific bystander effect (Fig 3A). This was assessed *in vivo* by implanting mixtures of RM1-CDUPRT and RM1-GFP cells Iprost in different proportions, followed by treatment with 5FC. Prostate tumor volumes measured at necroscopy indicated that the minimal proportion of RM1-CDUPRT cells required to produce a therapeutic effect was 20% ($p=0.01$) (Figure 3B).

Apoptosis after GDEPT: To investigate how GDEPT mediates cell death *in vivo*, the extent of apoptosis was evaluated by TUNEL assay (34) on tumors from different treatment groups (Fig 4A, Table2). Scattered apoptotic cells were seen throughout the tumors of RM1-LacZ/5FC or RM1-CDUPRT/saline controls. Apoptosis was markedly increased in tumors of the GDEPT groups in a dose dependant manner. An increase of 1.8 fold was seen in 100% RM1-CDUPRT/5FC group compared with 100%RM1-GFP/5FC group ($p=0.0001$), in both necrotic and non-necrotic areas.

Tumor vasculature after GDEPT: We noted in the previous experiment that CDUPRT-GDEPT tumors were characterised by extensive haemorrhagic necrosis suggesting that the treatment may have disrupted the tumor vasculature. To investigate this possibility, vascular analysis (anti-CD31 staining) of the entire tumor section was performed. Any stained endothelial cells or clusters separated from adjacent microvessels were included and counted as one microvessel, whereas infrequent CD31-positive macrophages and plasma cells were excluded from the analysis. Neither vessel lumens nor red blood cells were used to define a microvessel (18). CDUPRT/FC caused a reduction in the vascularity of the tumors by more than 3X compared to the control RM1-GFP tumors (Table 2, Fig. 4B, $p=0.006$). This extent of reduction increased with increasing number of CDUPRT expressing cells (Table 2) suggesting the vasculature disruption to be CDUPRT-GDEPT-specific and possibly involved in the enhancement of its cytotoxicity.

3. Distant bystander effects of CDUPRT-GDEPT

Next, we investigated if CDUPRT GDEPT leads to a similar '*distant bystander effect'* by assessment of whether killing of cells in the prostate by CDUPRT-GDEPT would have any effect on the growth of pseudometastases of the parental RM1 cells in the lung. Three independent experiments were performed and data from a representative experiment is shown. The number of lung colonies in mice in the RM1-CDUPRT/5FC group was much lower than those in the control RM1-GFP/5FC or RM1-CDUPRT/saline groups (Fig.4A and 4B). The lungs of the control groups were completely covered with RM1 tumors in all mice and this posed logistical problems. Therefore, they were arbitrarily given the value of 450 colonies (Fig. 4B) on the basis of the average of counts done in 3 representative mice from each control group. While similar trends were seen in all three experiments, there were variations in lung colony numbers between experiments. In one experiment, 50% of the GDEPT treated mice had no lung colonies compared with no such mice in the control groups (data not shown). This suggests that a '*distant bystander effect'* may have prevented the growth of RM1 lung colonies.

Immunohistochemical studies of RM1 tumors The distant bystander effect is characterized by infiltration by the immune cells e.g. macrophages, $CD8^+$ $CD4^+$ T, B and NK cells in tumors undergoing GDEPT (19-22). Immunoperoxidase staining was used to assess infiltration by macrophages, $CD8^+$ $CD4^+$ T, and NK cells in Iprost tumors from different treatment groups (Table 2). Increasing numbers of $CD4^+$ T cells were detected in all three RM1-CDPURT/5FC treatment groups (B, C and D) compared with RM1-GFP+5FC control group (A) ($p<0.05$). Further, this recruitment was enhanced by 7X in tumors from 100% RM1-CDPURT/5FC group compared with 2X in 20% and 10% mixed cell tumors (Fig 6A). In contrast, staining for $CD8a^+$ cells was minimal in all groups. Although not statistically significant, increasing numbers of infiltrating $F4/80^+$ (macrophages) and Asialo-$GM1^+$ (including NK) cells were detected in tumors of the three RM1-CDPURT+5FC groups (B, C) compared with the control group (A, Table 2); representative sections from treated tumors are shown in Fig. 6. This CDUPRT-specific dose dependant increase in $CD4^+$, macrophages and NK cells is a strong indication of the involvement of the immune system in local and distant bystander effects.

DISCUSSION

We previously reported that a single dose of PNP-GDEPT was effective against orthotopic RM1 tumors, leading to increased survival (17,23). We have now explored a new GDEPT system, CDUPRT fusion gene with the prodrug, 5FC. We wanted to examine the local and distant bystander effects of CDUPRT-GDEPT in the context of an intact immune system particularly as it was implicated in developing systemic anti-tumorigenicity with CD/5FC and UPRT/5FU GDEPTs (24,25). We used a syngeneic immunocompetent mouse model of orthotopic and pseudo-metastatic murine PC (26) which was further characterized by us (27). This model is susceptible to other GDEPTs (HSV/TK, PNP) (17,23,28). Further, RM1 cells are mildly immunogenic and express MHC class 1 molecules making them susceptible to immune system mediated cell killing (28,29). Further when tested for sensitivity to 5FU, sensitivity of RM1 cells (57% cell killing at 1μg/mL (7.7μM) was comparable to that of mouse mammary carcinoma (1μM) and mouse lymphoma cells (10μM) (30). This was higher than the accepted therapeutic range in humans and offered an ideal therapeutic window for assessment of CDUPRT GDEPT. When tested for 5FC sensitivity, the control RM1LacZ cells were not affected even at 100μg/mL of 5FC but efficient cell killing was seen for RM1-CDUPRT cells at low doses of >3μg/mL, well within the accepted steady state levels in humans (50μg/ml) (31). The 5FC related systemic toxicities were undetectable in mice with no loss of condition at the experimental dose (500 mg/kg/mouse) commonly used in other studies (6).

The *in vivo* efficacy of the CDUPRT GDEPT against RM1 Iprost tumors was unequivocally proven and supports other studies showing the efficacy of the combination against different types of cancers including PC (6,7,13). Treatment was associated with a local bystander effect (Fig 3), and stimulated a distant bystander effect as evidenced by inhibition of pseudo-metastases in lungs (Fig 5). *This is the first in vivo demonstration of local and distant bystander effects in PC using the fusion gene, CDUPRT*. Bystander effects of CDUPRT GDEPT are operated via two independent mechanisms: diffusion of cytotoxic 5FU and transfer of the cytotoxic fluoronucleotides (FUMP and FUTP) via intercellular gap junctions (ICGJ) to surrounding cells. This provides for the cell type-based variation in the bystander effects. Indeed, *in vitro* local bystander effects caused by CDUPRT-GDEPT are described for glioma (6), glioblastoma (11) and colon carcinoma (7), generally when 2- 10% of cells expressed the transgene. This variability was due to differences in 5FU sensitivity (11) and ICGJ status of the cells (6). Miyagi *et al* (13) showed that ICGJ lacking DU145 human PC cells showed only 30% cell killing when 10% cells were expressing CDUPRT, *in vitro*. While CDUPRT- GDEPT was more effective than CD-GDEPT against DU145 xenografts, the local bystander effects for these two GDEPTs against DU145 cells showed an opposite trend *in vitro*. The authors postulated that although the active phosphorylated metabolites of the CDUPRT GDEPT could not diffuse efficiently in DU145 cells which lack ICGJ (12), a more efficient conversion of 5FC into 5FU sensitized the cells to 5FU leading to enhanced anti-tumor killing *in vivo*.

In our study, we demonstrated the presence of the local bystander effect *in vitro* qualitatively. Use of cell mixtures containing different proportions of RM1 cells with RM1-GFP-CDUPRT cells did not yield significant data as the RM1 cells grow twice as fast compared with RM1CDUPRT cells both *in vitro* and *in vivo* (data not shown). Interestingly, when investigated *in vivo,* the data clearly showed a bystander effect when 20% of the cells expressed the transgene. This was significant given that the faster growth rate of RM1 cells would bias the results against the demonstration of the bystander effects. Other factors that could affect the extent of bystander effect in our system: A. Moderate sensitivity of the RM1 cells to 5FU toxicity (7.7 μM compared with 17μM in 5FU resistant DU145 cells) B. Diminution of the pool of available 5FU due to efficient conversion to its metabolites (4) C. RM1 cells lead to aggressive non-differentiated tumors *in vivo* (27) and it is known that loss of ICGJ is a critical step in progression to human prostate neoplasia (32). It is likely that intercellular gap junctions are absent or present at low concentration in RM1 tumors and hence the bystander effects are not as dramatic as expected from other studies (6,7).

Our laboratory is the first to describe the distant bystander effect of CDUPRT-GDEPT in vivo. We have shown that operating the CDUPRT-GDEPT in the prostate of mice results in a considerable suppression of parental RM1 colony formation in the lungs.

Two factors may contribute to this. The first is due to chemically induced responses related to RM1-sensitivity to systemically given 5FC or 5FU introduced into the bloodstream during GDEPT. It was unlikely that 5FC contributed to toxicity as RM1 cells were resistant to 5FC even at 100μg/mL and serum and histological analysis of organs from mice given 5FC did not show any 5FC related toxicity; also 5FC or 5FU could not be detected in the blood sera of the treated mice. This suggested an alternate mechanism such as anti-tumor immune responses triggered during GDEPT may have mediated cell killing of RM1 cells. It was postulated (33) that a distant bystander effect is induced due to release of cytokines via immune cell activation, triggered by GDEPT induced tumor destruction. This leads to hemorrhagic necrosis that then allows more immune cells to infiltrate the tumor. A number of studies support this theory (reviewed in (1). Our studies show that immune cells infiltrating the primary tumor included $CD4^+$T cells, macrophages and NK cells, suggesting their involvement in anti-tumor activity in this system. This is a wider repertoire of immune cells compared to when either GDEPT is used alone. The distant bystander effects from CD- and UPRT-GDEPTs are mediated by the immune system

(14,15,34). Thus UPRT expressing murine colon carcinoma cells in syngeneic immunocompetent mice led to tumor regression when treated with 5FU compared with wild type tumor cells (14). Treated mice rejected the wild type- but not irrelevant syngeneic tumor cells. This distant bystander effect was less efficient in nude mice suggesting that αβ T cells were involved. In comparison, in a rat model that mimics liver metastases of colon carcinoma, CD GDEPT led to regression of CD positive tumors (25,34) and resistance in treated rats to wild type challenge. Immunodepletion studies showed that NK cells were involved. This would suggest that NK and αβ T cells are implicated in CD and UPRT GDEPT effects, respectively. Hence, when the two systems are combined the immune system may be augmented by the inclusion of a larger repertoire of anti-tumor immune cells. Our preliminary analyses support this, but immunodepletion studies are needed to ascertain the mechanisms involved.

Hemorrhagic necrosis was a remarkable feature of CDUPRT/5FC treated RM1-GFP/CDUPRT tumors (Fig 2C,Table 1) suggesting that endothelial cells in the tumor vasculature were susceptible to GDEPT. This disruption could be mediated by the cytokines released due to the stimulation of the immune system (33) or due to the toxicity of 5FU released in the tumor microenvironment (35). G1-arrest in endothelial cells was observed when treated with 5FU (39). In our study, haemorrhagic necrosis increased in a dose dependant manner with GDEPT treatment (Table 2), strongly suggesting that disruption of tumor vasculature may have contributed further to the tumor regression in CDUPRT treated mice. The elucidation of the mechanism of this disruption however, was beyond the scope of this study.

Killing of tumor cells by anticancer therapies such as chemo-, radiation-, immuno- or suicide gene therapy is predominantly mediated by triggering apoptosis. Our data indicated that apoptosis was involved in the death of RM1 tumor cells expressing CDUPRT (Fig. 5) and again the dose dependence of this effect suggested that it was CDUPRT GDEPT-specific. This accords with other studies showing the involvement of apoptosis in cell killing mediated by various GDEPTs such as HSV/TK, PNP and CD (23,36-38).

Although the CDUPRT-GDEPT has not yet been trialled in humans, the safety of the CD-GDEPT has been well reported in PC patients (39). In this preclinical study, there was no apparent toxicity to other organs indicating that CDUPRT-GDEPT has excellent safety features against normal host tissues. There is increasing emphasis on use of gene therapy in concert with other strategies, including strategies that enhance anti-tumor immunity. Our data indicate that CDUPRT/5FC GDEPT significantly suppressed the growth of RM1 cells producing both a local and a distant bystander effect by mechanisms of killing that involve necrosis and apoptosis possibly mediated by the immune responses. This proof of principle study will form the basis of the future studies assessing the impact of combination of CDUPRT GDEPT with immunotherapy.

1. MATERIALS AND METHODS

Cell lines and mice

The RM1 (16) and the transformed derivatives (RM1-CDUPRT, RM1-GFP and RM1-LacZ) were cultured in Dulbecco's Minimal Essential Medium (DMEM) (Invitrogen, CA, USA) containing 10% fetal calf serum (Invitrogen, CA, USA) with hygromycin (hygro) (Invitrogen, CA, USA) at a concentration of 800μg/ml for the later. Male C57BL/6 (6-8wk) mice were bought from Labortory Animal services, Perth, AU.

Transfection of RM1 cells

RM1 cells were transfected with pVITRO2-GFP/CDUPRT, pVITRO2-GFP/LacZ (Invivogen, CA, USA) or pVITRO2-GFP (InvivoGen, CA, USA) to generate stable transfectants (RM1-CDUPRT, RM1-LacZ and RM1-GFP). The pVITRO2-GFP/CDUPRT was constructed by

excision of CDUPRT (CodA::upp) gene from pORF-codA::upp (InvivoGen, CA, USA) using *NcoI* and *NheI* restriction enzymes followed by its ligation into complementary sites in the pVITRO2-GFP/LacZ (InvivoGen, CA, USA). The construct was authenticated by restriction enzyme digestion using *NcoI* and *NheI*. For transfections, cells were transfected with complexes formed by combining 15µl Lipofectamine 2000 (Invitrogen, CA, USA) and 5µg plasmid DNA according to manufacturer's instructions. Stable clones were maintained under hygro B selection (800µg/mL). GFP expression was used to sort the cells (FACscan sorter, BD, USA) for high levels of GFP expression and to eliminate drug-resistant, non-expressing clones.

Optimization 5FC dose *in vivo*

Mice injected ip with 150, 300 and 500 mg/kg/mouse/day of 5FC (InvivoGen, CA, USA) or saline for 13 days were monitored daily for general behaviour and condition and their body weights were measured every second day. Toxic effects were monitored by assessment of liver and renal function via evaluation of the levels of urea, creatinine, alkaline phosphatase (ALP), alanine amino-transferase (ALT) and aspartate amino-transferase (AST) in the serum samples by the South East Area Laboratory Services (Prince of Wales Hospital, AU) using standard techniques.

Assessment of CDUPRT expression *in vitro* and *in vivo*

To assess the functionality of CDUPRT in RM1-CDUPRT cells/tumours, an HPLC based assay was developed measuring the catabolism of the prodrug 5FC to 5FU. Homogenates of RM1-CDUPRT or –LacZ cells sc/intraprostatic (Iprost) RM1-CDUPRT or –LacZ tumors (Liquid N_2) were generated and lysis was completed by 3 cycles of freeze thawing. Cell debris was removed by centrifugation (15,000g, 10 min) followed by determination of the protein content of the supernatants (BCA protein estimation kit, Pierce, IL, USA). 100µL of the supernatant was then incubated with 900 µL of 0.5 mM 5FC at 37^{o}C. At 24 h, the samples were stored at –20^{o}C, after a 10 min incubation at 85^{o}C. Reversed phase liquid chromatography employing a C18 column under isocratic conditions (0.05% Trifluoroacetic acid in H_2O) at a flow rate of 0.7 mL/min was used to analyse samples (10 µL). Absorbance was measured at 275 nm. The enzyme activity for each sample was determined by ratios of the peak areas for 5FC and 5FU.

GDEPT *in vivo*

Iprost injections were performed with $5x10^3$ RM1-CDUPRT (test) or RM1-LacZ (control) cells in the subcapsular region of the prostate surgically after opening the abdomen in C57BL/6 mice as previously described (23). Day 4 onwards, 5FC or saline was administered ip at 500 mg/kg/day for 14 days. Mouse weights were recorded twice/week. At necroscopy (day 18), the prostate tumor volumes were determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$ where d_1 and d_2 are diameters at right angles (40). The tumor and other organs (kidney, lungs, liver, heart, spleen) were fresh frozen or paraffin-embedded for subsequent histological and immunohistochemical studies. Mouse serum was stored at –80^{o}C until analysis.

Local bystander effect on RM1 growth

In vitro: Conditioned media from RM1-CDUPRT cells +/- 5FC (at 1mM) or RM1-LacZ cells+5FC were harvested 48 h after the addition of 5FC. These mixed with an equal proportion of fresh medium were then incubated with RM1 parental cells ($5x10^3$ cells/well in a 96 well plate). Cell viability was determined at 72 h using the WST1 proliferation assay (Roche, Sydney, AU) according to the supplier's instructions.

In vivo: RM1-CDUPRT cells and RM1-GFP cells were mixed in different proportions and implanted Iprost in mice followed by 5FC treatment daily for 14 days. As data from our earlier experiments showed the GDEPT to be specific to RM1-CDUPRT/5FC group, to minimize the mouse usage, the saline controls included earlier were not performed. On day 19, tumors and other organs were processed as described above.

Effects of RM1-CDUPRT plus 5FC on growth of pseudometastases in the lungs

Mice were injected Iprost with $5x10^3$ RM1-CDUPRT or RM1-GFP cells. On day 4, mice received $2.5x10^5$ RM1 cells iv and 5FC treatment daily ip for 15 days. On day 19, their lungs were fixed in Bouin's fixative and the RM1 colonies were counted as previously described (23).

Immunohistochemical analysis of orthotopically implanted prostate tumors

For detection of Immune infiltration: Snap frozen tissues were embedded in Optimal Cutting Temperature compound (Tissue Tek), sectioned (5 μm) and acetone fixed. To block endogenous peroxidase/biotin and non-specific monoclonal antibody binding, sections were incubated sequentially with 1.5% H_2O_2 (5 mins), avidin block (10 mins), biotin block (10 mins), 3% bovine serum albumin in PBS (5 mins). Sections were then stained using rat α mouse -CD4 (BD-PharMingen, 1:100); -CD8a (BD-PharMingen, 1:200); -F4/80 (BD-PharMingen,1:800), -CD31 1:300, BD-Pharmingen) and rabbit α mouse AsialoGM1 (Dako,1:400) as relevant by incubating for 45 minutes at room temperature followed by incubation with secondary antibodies, α -rat (1:200) and α-rabbit (1:200) and the ABC complex for 15 minutes. The standard ABC detection system, the diaminobenzidine (DAB) as the chromagen and Harris hematoxylin as counterstain were used.

For detection of Apoptosis: Tissues fixed in 10% neutral buffered formalin (Amber Scientific) were paraffin-embedded (Tissue Tek-VIP (Sakura). Dewaxed paraffin sections (Histochoice, Ambresco) were rehydrated through graded series of ethanol and finally in PBS before blocking. Apoptotic cells were detected using the Tunel assay kit (Roche) as described (41) after antigen retrieval.

Scoring of positive stained cells in the immunostained sections was performed by light microscopy. After initial scanning under x100 magnification, positive stained cells in ten fields under x400 (0.15 mm^2) magnification were counted and the mean number/high power field (HPF ± SEM) was determined. Because of the high number of positive apoptotic cells, a higher magnification (x620) was used.

Data analysis

A one-way analysis of variance (ANOVA) was performed (GraphPad PRISM V4) if the data in multiple groups were normally distributed. A Turkey's post-test was performed if the ANOVA indicated a significant difference ($p<0.05$) between treatments.

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Table 1: Effects of CDUPRT-GDEPT on prostate histology.

Treatment 5 x 10^3 RM1-GFP/CDUPRT (Iprost)	Viability	Necrosis	Haemorrhagic[a] Necrosis
+ saline ip 15 days (n=6)	>80%	<10%	<10%
+ 5FC (500 mg/kg/day) ip daily for 15 days (n=4)	<10%	>30%	>60%
+ 5FC (500 mg/kg/day) ip daily for 15 days (n=6)	No	No growth	No growth

[a] Haemorrhagic necrosis was defined by necrotic cell death observed in areas with vascular damage, primarily observed in tumours treated with CDUPRT-GDEPT.

Table 2. Immunohistochemical analysis of infiltrating immune cells, endothelial cells (vascularity) and apoptotic tumor cells in RM1-GFP/CDURPT+5FC and control tumors.

Treatment	Number of Infiltrating immune cells								Vasculature (endothelial cells)		Apoptotic tumor cells	
	$CD4^+$		$CD8a^+$		$F4/80^+$		$AsialoGM1^+$		CD31		Tunel assay	
	Mean[a]	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
(A) RM1-GFP+5FC (Control)	4.55	0.39	1.26	0.40	10.85	1.90	13.05	1.10	18.03	2.89	47.03	3.74
(B) 10%RM1-GFP/CDURPT mixed with 90%RM1-GFP +5FC	9.43	3.36	4.25	1.27	20.53	0.76	14.93	1.82	13.82	2.09	65.30	0.62
(C) 20%RM1-GFP/CDURPT mixed with 80% RM1-GFP +5FC	9.40	3.46	1.73	0.90	16.23	2.38	16.20	2.76	8.33	0.13	72.97	2.17
(D) RM1-GFP/CDURPT (100%) +5FC	32.9	8.9	1.70	1.70	22.00	4.71	20.27	3.94	5.67	0.35	84.25	0.75
One way ANOVA P value	0.003		0.19		0.002		0.04		0.0061		0.0001	

[a] Mean number of positively stained cells/high power field (HPF $\pm$ SEM) (x400) (0.15 mm square) from a count of 10 fields. SEM: Standard error of the mean.

Figure Legends

Figure 1: Analysis of toxicity of 5FC in C57BLl/6 mice. Mice (4 mice /group) were injected ip with 100, 150 and 500 mg/kg/day of 5-FC or saline for 13 days. At necroscopy, heart, liver, lung and spleen were harvested and analysed by H&E staining for toxic effects of the drug. (A)The four panels show different organs from mice treated at the highest dose, 500mg/kg/day. Insets in each panel represent the corresponding organ from control mice treated with saline. There was no detectable toxicity even at 500mg/kg/day. (B) serum analysis for biochemical markers of kidney and liver function (ALP: Alkaline Phosphatase, ALT: Alanine Amino Transferase and AST: Aspartate Amino Transferase is shown. The reference represents the values for a normal mouse.

Figure 2: *In vivo* evaluation of therapeutic effects of CDUPRT-GDEPT using RM1-GFP/CDUPRT cells +5FC

(A) 5x10^3 cells were implanted orthotopically in the prostate of C57BL/6 mice. 4 days post-implantation the prodrug 5FC or saline were administered ip at 500mg/kg/mouse/day for 13 days. At necroscopy (day 17), prostate tumor volume was measured. Prostate volumes were determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$, where d_1 and d_2 are diameters at right angles.

(B) H&E staining of paraffin-embedded orthotopic RM1-CDUPRT prostate tumor sections show that treatment with saline resulted in highly vascularized viable tumor (x40), (C) Treatment with 5FC resulted in extensive necrosis (black arrow) and haemorrhagic necrosis (red arrow) (C x40), with some loss of prostate tissue architecture (insert, x10).

Figure 3: Evaluation of "local bystander effect" of CDUPRT *in vitro* and *in vivo.*

(A) *In vitro*: RM1-GFP/CDUPRT cells were grown in presence or absence of 5FC for 48 h, then the supernatants were collected. Supernatants (conditioned media, CM) from RM1-GFP/LacZ cells grown in the presence of 5FC, served as controls. Parental RM1 cells were then treated with these CMs at 50% concentration. The bystander effect was demonstrated by cell killing of parental RM1 cells using theCM from RM1CDUPRT cells using WST1 viability assay (see methods).

(B) *In vivo*: Local bystander cell killing effects of the CDUPRT suicide gene is demonstrated. RM1-GFP/CDUPRT cells were mixed with RM1-GFP cells in different proportions and 5x10^3 total cells were implanted in the prostate of C57BL/6 mice. The mice were injected intraperitoneally with the prodrug, 5-fluorocytosine (5FC) at 500 mg/kg/day, from day 4 onwards

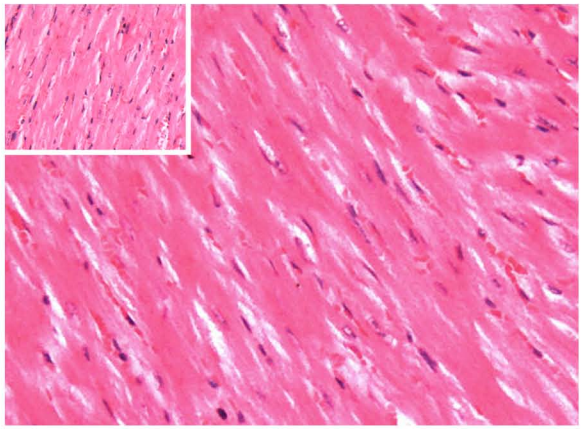
daily for 15 days. Mice were sacrificed on day 19, and their prostate volumes determined using the formula, $V = \pi/6(d_1.d_2)^{3/2}$, where d_1 and d_2 are diameters at right angles.

Figure 4: Assessment of apoptosis and vascular integrity in test (CDUPRT + 5FC) or control (RM1-GFP + 5FC) tumors (all x40): (A) Tunel positive cells in test tumor vs Inset, Tunel stain control tumor; (B) Decrease in CD31 positive cells in RM1-GFP/CDUPRT + 5FC tumor vs Inset, CD31 positive cells in control tumor.

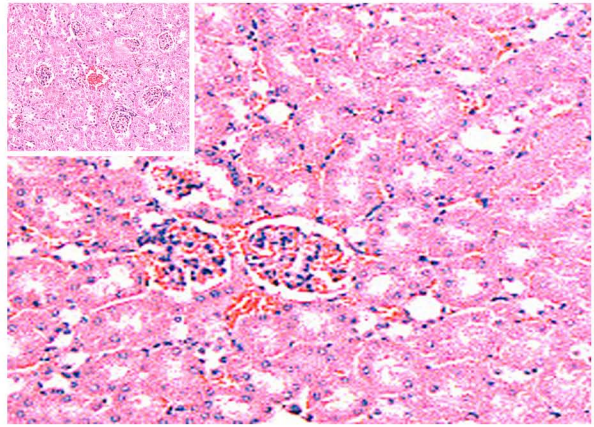
Figure 5: Distant bystander effect of CDUPRT suicide gene *in vivo*. RM1-GFP/CDUPRT cells ($5x10^3$) were implanted in the prostate of C57BL/6 mice. Four days later, the mice were injected intravenously with the parental RM1 cells at the dose of $2.5x10^5$ cells/mouse. The mice were injected with the prodrug 5FC from day 4 onwards daily for 15 days. At necroscopy, the lungs were harvested, stored in Bouin's reagent and colony counts were performed. (A) Scatter graph showing the number of lung colonies in different treatment groups (B) Photographs of lungs demonstrating the distant bystander effect of CDUPRT GDEPT on pseudometastases.

Figure 6: Evaluation of immune cell infiltration after treatment in test (CDUPRT + 5FC) or control (RM1-GFP + 5FC) tumors (all x40) (A) Cluster of CD4 positive T cells (brown colour) in test tumor; Inset few CD4 positive T cells in control tumor.; (B) Cluster of F4/80 positive macrophages in test tumor. vs Inset: control tumor; (C) Asialo-GM1 positive immune cells clustered at the tumor periphery in test tumor vs Inset, Control tumor.

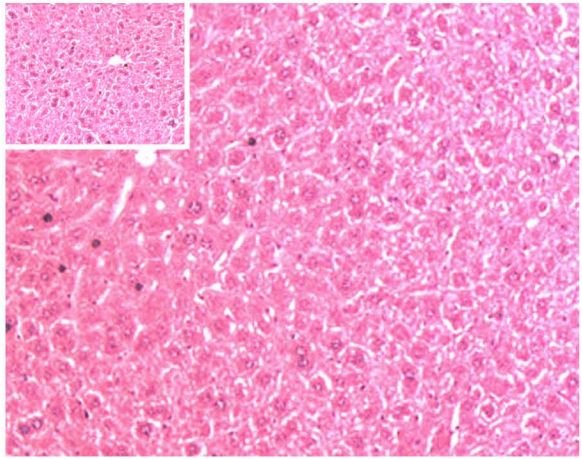
A



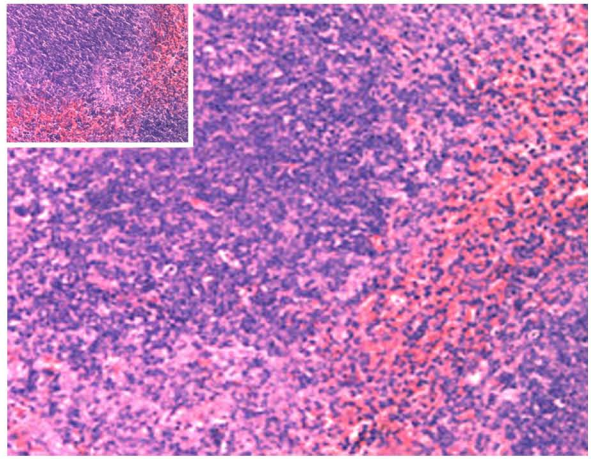
Heart



Kidney



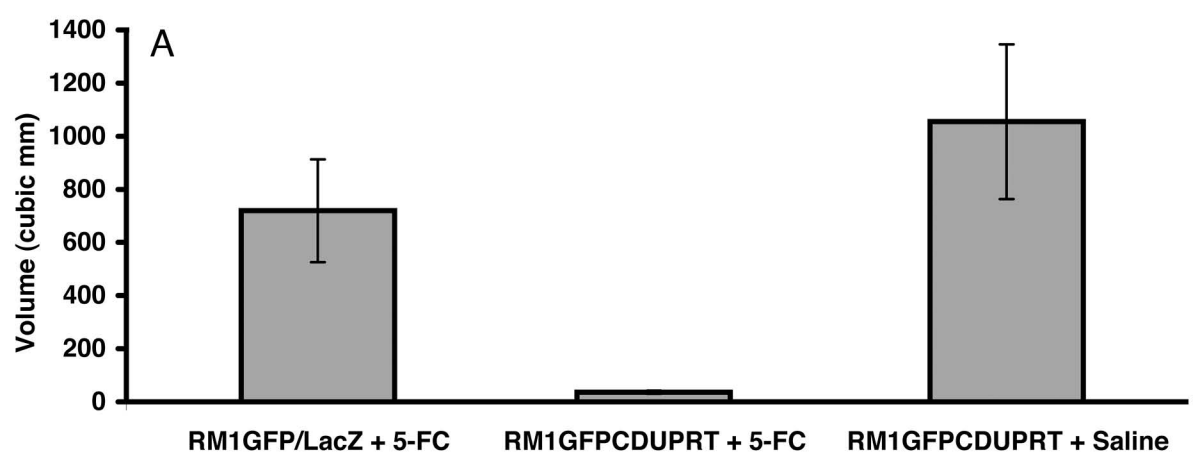
Liver

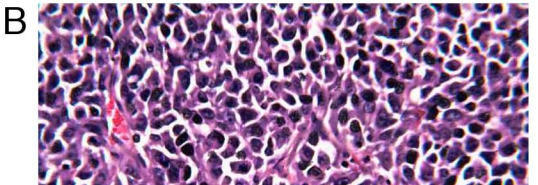


Spleen

B

Treatment	Urea (mmol/L)	Creatinine (umol/L)	ALP (U/L)	ALT (U/L)	AST (U/L)
Reference	**1.4-5.5**	**18-80**	**35-96**	**17-77**	**54-298**


A
1400
1200
1000
800
600
400
200
0
Volume (cubic mm)
RM1GFP/LacZ + 5-FC
RM1GFPCDUPRT + 5-FC
RM1GFPCDUPRT + Saline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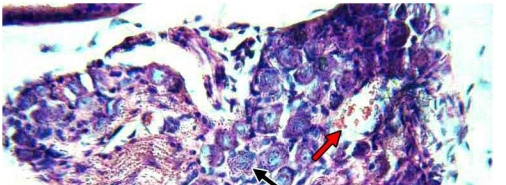
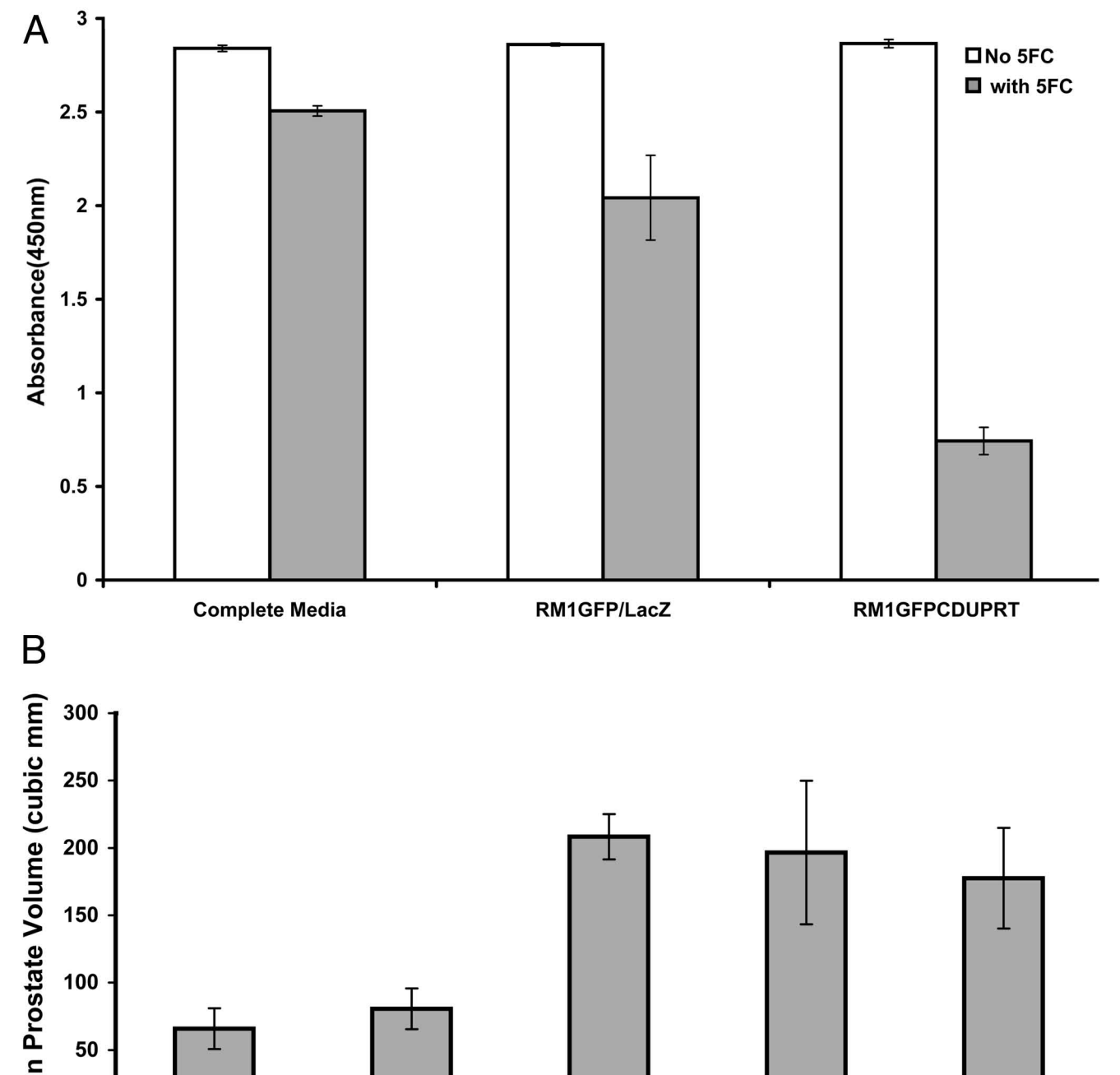

C


A
Absorbance(450nm)
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2.5
2
1.5
1
0.5
0
No 5FC
with 5FC
Complete Media
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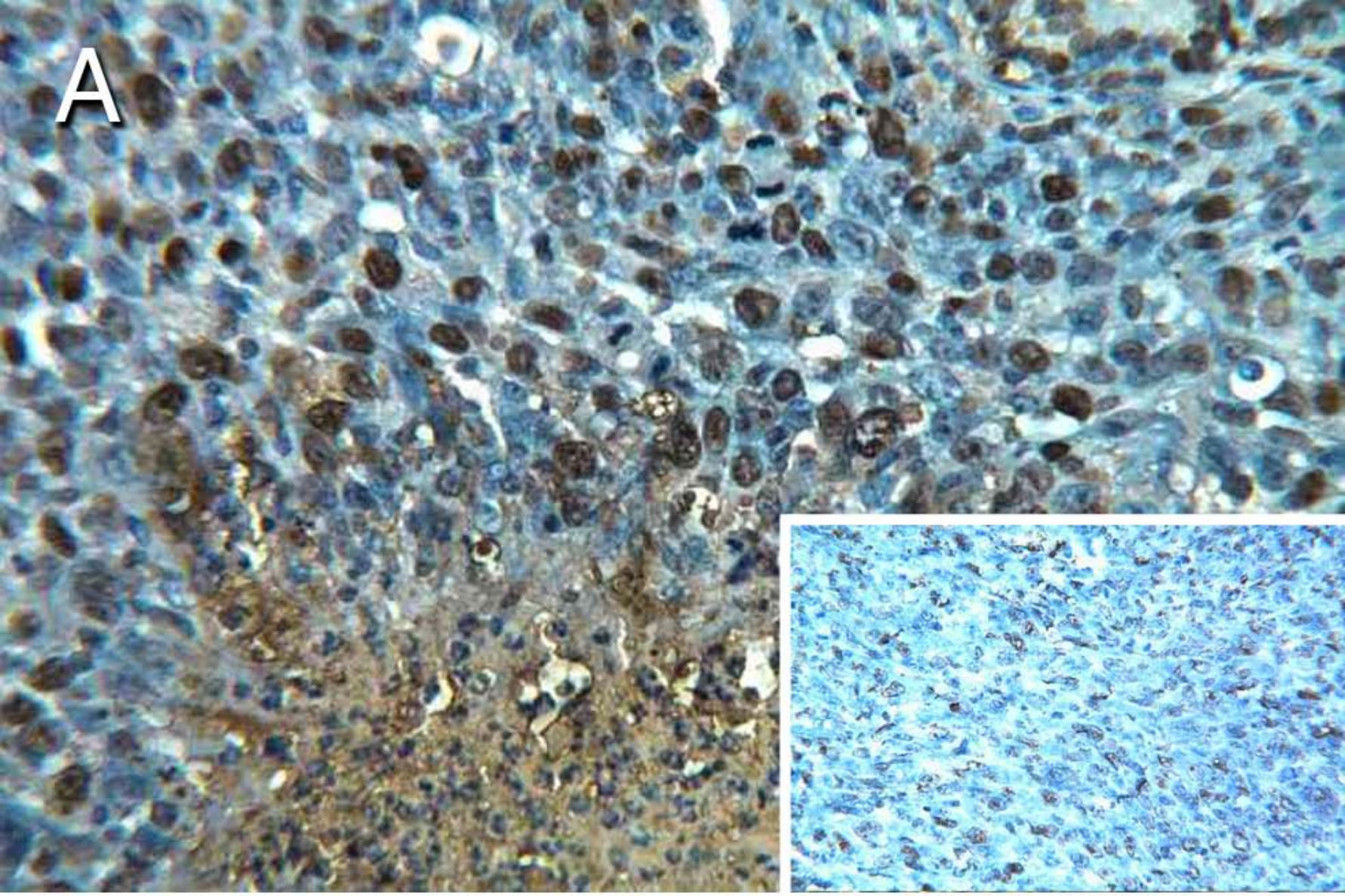
n Prostate Volume (cubic mm)
300
250
200
150
100
50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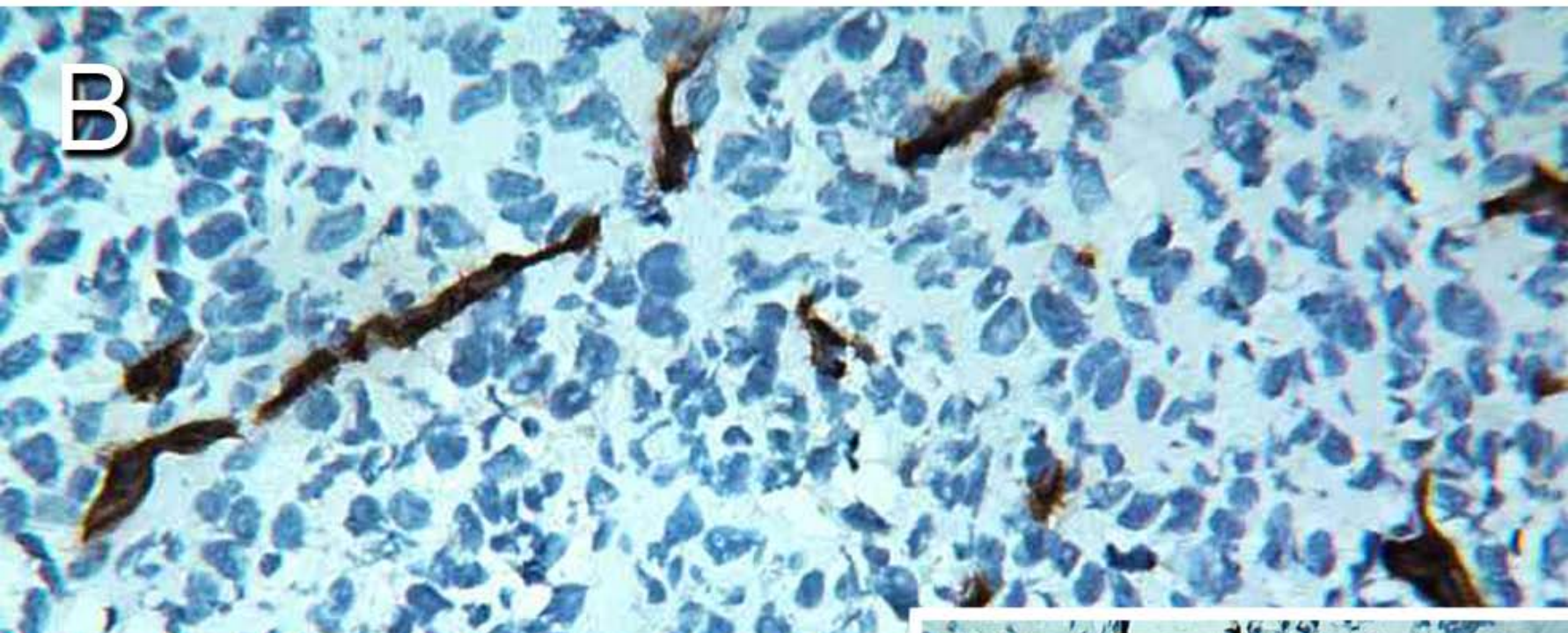
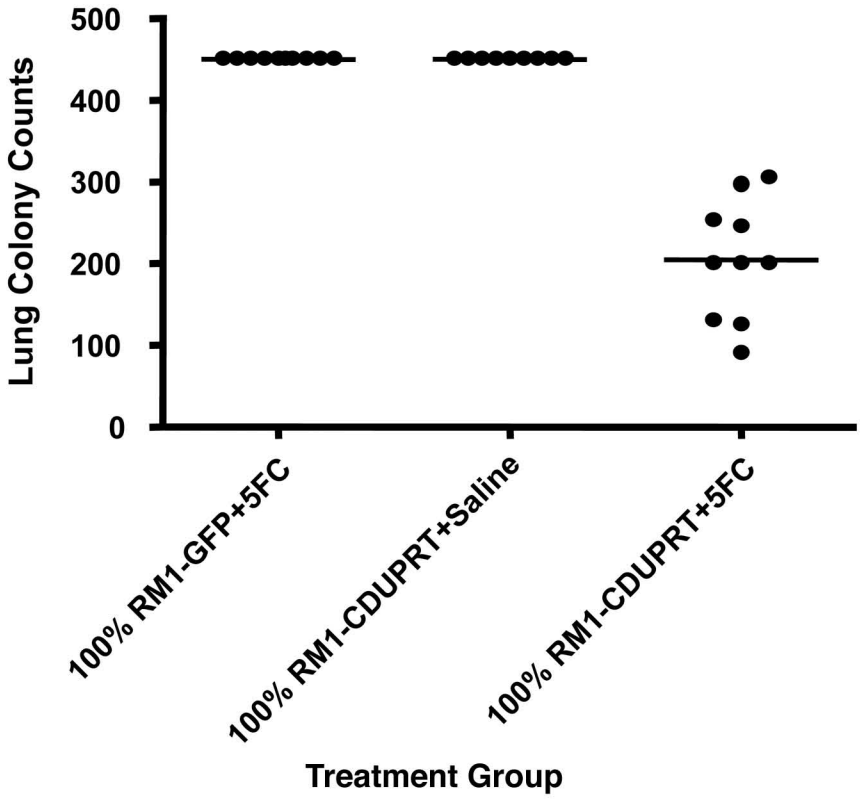
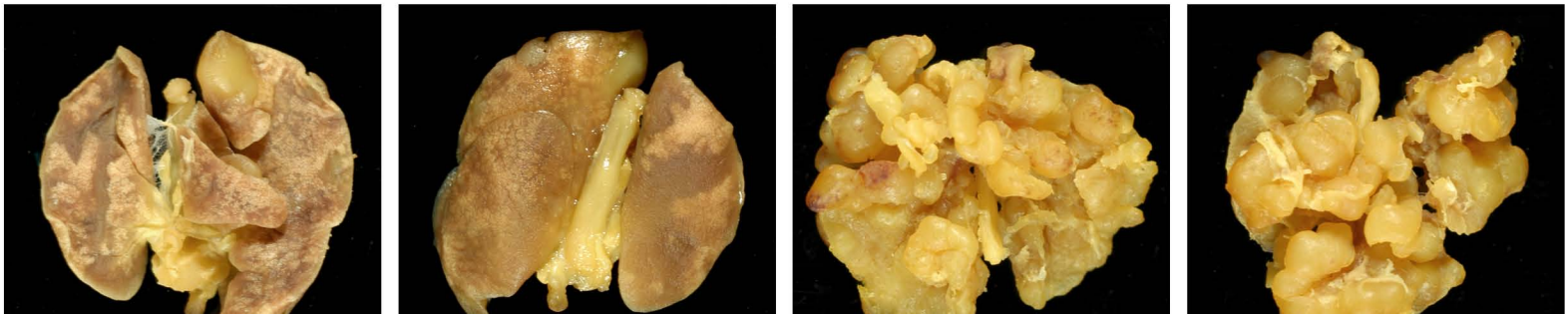
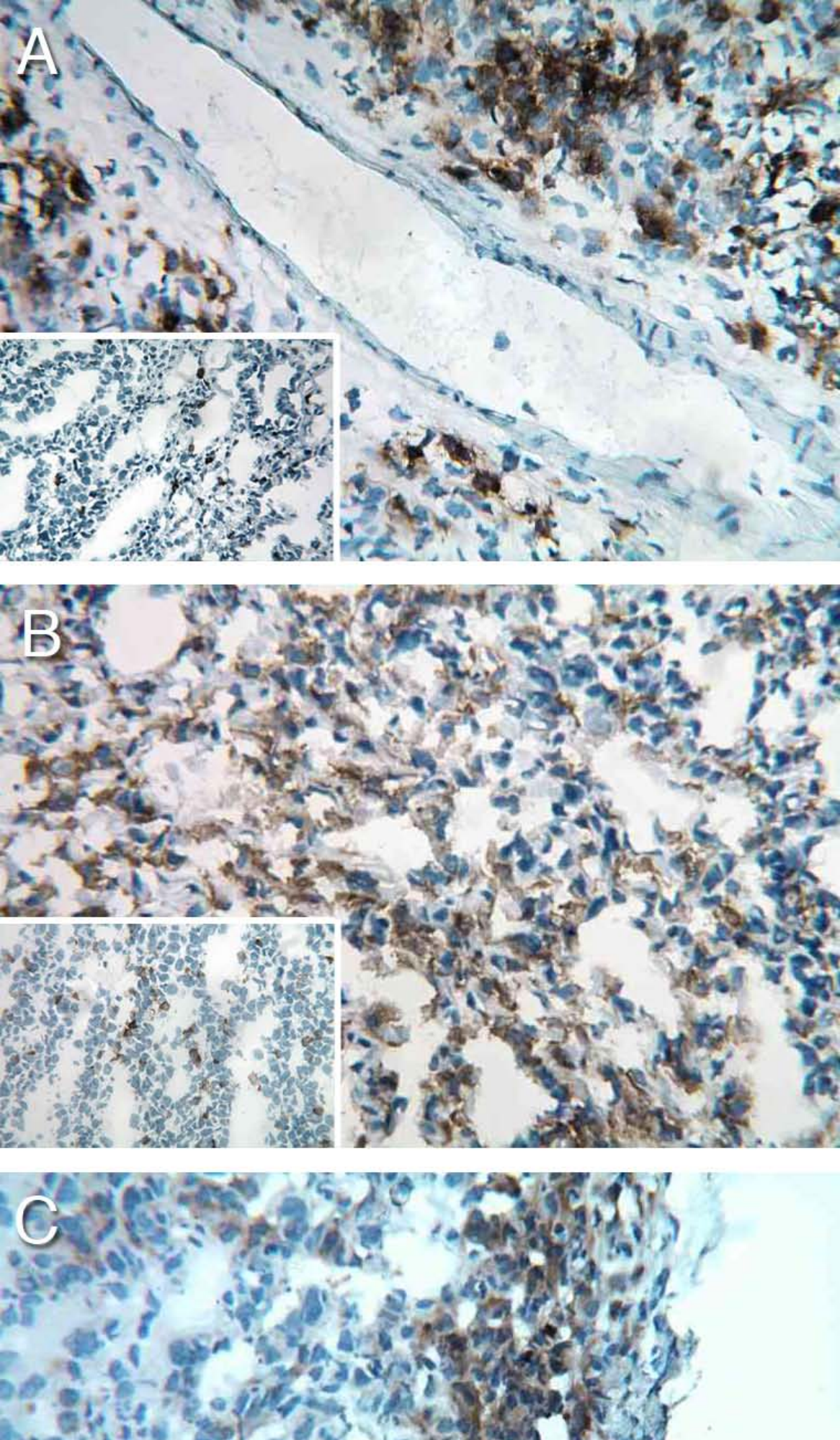

B

A


Lung Colony Counts
500
400
300
200
100
0
100% RM1-GFP+5FC
100% RM1-CDUPRT+Saline
100% RM1-CDUPRT+5FC
Treatment Group

B




A
B
C

Editorial Manager for Journal of Immunological Methods

Manuscript Draft

Title: **A novel cell line based bioassay for measurement of murine cytokines IL12 and IL18.**

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Running Title: A novel cell line based bioassay for measurement of murine cytokines.

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Abbreviations Used:

A novel cell line based bioassay for measurement of murine cytokines IL12 and IL18.

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INTRODUCTION:

Research in the cytokine field has increased tremendously because not only are they involved in the production, maintenance and function of the haematopoietic/ immune system, but they have shown immense potential for therapy of a wide range of diseases including cancer (Dinarello *et al.*, 1989; Hirano *et al.*, 1990; Tracey and Cerami, 1993; Dinarello, 2005). The detection of cytokines in various biological fluids and laboratory samples is a necessary aspect of that research. There are two types of assays that can be used- Immunoassays and Bioassays. While immunoassays are easy to carry out, they can lead to inter-assay variation depending on the format and reagents used for analysis. The advantage of bioassays is that the ability to assess the presence of biologically active cytokine unlike the immunoassays, which measure both active and inactive cytokine (Mire-Sluis *et al.*, 1995). A bioassay typically measures the biological activity of specific cytokine/s, which is more relevant to the *in vivo* action of that cytokine (Mire-Sluis *et al.*, 1995). Generally, bioassays employ the use of purified haematopoietic cells (from blood or bone marrow) or continuous cell lines (reviewed in (Mire-Sluis *et al.*, 1995). While the use of purified haematopoietic cells for cytokine bioassays can provides a quantitative estimate of cytokine levels, these assays are still subject to donor variation, which makes

the inter-assay comparisons difficult to interpret. In addition, purified cell preparations are often contaminated with other cell types, which can skew the results and add to the inter-assay variability. On the other hand, continually growing cell lines or receptor-transfected cell line based bioassays are more specific, easier to perform and avoid inter-assay variability thus generating more accurate data. One of the most important animal ethics requirements is to minimise the numbers of animals for experimental purposes; hence the development of a cell line based biosassay that would minimise the use of animals as a substitute for *in vivo* work, would be very desirable.

In this study, we have attempted to develop a cell line based bioassay for detection of the murine cytokines, IL12 and IL18. Both IL12 and IL18 are Th1 type immunostimulatory cytokines that have shown immense potential as potential therapeutic agents for treating various types of cancers (reviewed in (Liebau *et al.*, 2002; Tatsumi *et al.*, 2003; Yamanaka *et al.*, 2003; Liebau *et al.*, 2004; Li *et al.*, 2005). Importantly, IL12 and IL18 synergize with each other by upregulating each other's receptors. IL-12 can upregulate the production of the IL-18 receptor α (IL18Rα) chain on Th1 cells (Ahn *et al.*, 1997) whereas IL-18 has been shown to upregulate the IL12Rβ2 chain on Th1 cells (Chang *et al.*, 2000). This shared upregulation of receptors provides a positive feedback mechanism allowing these cytokines to act synergistically. This synergism has been particularly effective in treatment of different types of cancer and the therapeutic effects are significantly higher than when either cytokine was used alone. As a result a number of groups (including us) are evaluating the combination of the two cytokines in the treatment of various types of cancers. While there are well-established assays that measure IFN-γ production by T cells in response to these cytokines, these employ the use of T cells purified from animal donors. We have attempted to develop a cell line based bioassay that was equally

sensitive and also capitulated on the synergistic interaction between the two cytokines that could hence be used to estimate both simultaneously in a biological sample.

MATERIALS AND METHODS:

1.Cell lines: CTLL2 cells (obtained from Dr Peter Williamson's laboratory from S Schibeci at Millennium Institute at Westmead Hospital, Sydney) are derived from C57BL/6 mice and grow indefinitely in culture in the presence of murine IL2 (mIl2)(Roche) (Baker *et al.*, 1979). These were routinely cultured in DMEM containing 10% Fetal calf serum, 20 mmol/L glutamine, 0.039 mM 2-mercaptoethanol (Sigma) and 11mM Na-pyruvate (Sigma) and 20u/mL of mIL2.

2. Antibodies and cytokines: Recombinant murine IL12 (R&D systems, USA) and mIL18 (Medical and Biological Laboratories Co., LTD, Japan) were used in this study. The following antibodies were used in this study; Anti murine IL12 (αmIL12, polyclonal goat IgG, R&D, USA), anti-murine IL18 (αmIL18, Medical and Biological Laboratories Woburn, MA, USA), biotinylated anti-mIL12 receptor antibody (BD Pharmingen, USA), anti mIL18 receptor antibodies (BD biosciences, USA) and anti CD3-FITC (BD Pharmingen, USA).

3. Staining of CTLL-2 cells for evaluation of mIL12 and mIL18 receptors: Cells ($2x10^5$/tube) were harvested and washed twice in PBS containing 2% FCS (PBS/FCS). This was followed by the staining of the cells with the primary antibodies, biotinylated anti-mIL12 receptor antibody (0.25μg/tube) or anti-mIL18 receptor antibody (1μg/tube) for 30 mins on ice. Cells were washed twice with PBS/FCS and incubated with the secondary antibodies, streptavidin-PE (1μg/tube) BD Pharmingen, USA) or anti goat-FITC (1μg/tube) for anti-mIL12 receptor or anti-mIL18receptor staining, respectively, for 30 mins on ice. For negative controls, cells were incubated with equal amounts of non-

specific isotypes, IgG2a-biotin (for mIL12R) and goat IgG (for mIL18 R) antibodies or secondary alone. After staining, cells were washed and resuspended in PBS for analysis using flow cytometry using a fluorescence based cell scanner (FACscan, BD).

3. Proliferation assay for CTLL2 cells: CTLL2 cells $5x10^3$ cells were seeded in a 96-well plate and cell proliferation was measured at different times by 4 h incubation of the cells using a calorimetric assay for quantifying the cell proliferation and cell viability, based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenase in viable cells (10µL/100µL, Roche Molecular Biochemicals, USA**).** Similarly, the CTLL-2 cells were tested for their proliferative responses to mIL12 and/or mIL18, at different doses, using the WST-1 based assay. For tritiated thymidine incorporation experiments, $2x10^4$ CTLL-2 cells (in 200µL volume) were treated with different doses of IL2 and 24H later 0.5µci of tritiated thymidine was added to each well. After 8h incubation the cells were harvested on to a silica filter using an automated cell harvester. The membrane was dried and the counts were done using the soft ware Top Count'.

4. Functional quantification of the cytokines using the splenocytes: The functional activity of the recombinant mIL12 and mIL18 cytokines was evaluated by their ability to induce IFN-γ production from activated T cells. Spleens were obtained from C57Bl/6 mice and stored on ice until the extraction of splenocytes (no more than 1h). A single cell suspension of the splenocytes was obtained by teasing the chopped mouse spleen through a 100 µ metal sieve with the aid of a syringe plunger. The cells were harvested and washed twice in PBS containing 5% fetal calf serum (FACSWASH). The T cells were isolated from these cells using αCD3 antibody using the magnetic bead based MACS anti-FITC beads (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) according to the supplier's instructions. Briefly, 10^8 cells suspended in 1 mL of FACSWASH were

incubated with 30 μL of ∝CD3-FITC primary antibody for 1h on ice. The cells were washed three times in FACSWASH and incubated in 1 mL of FACSWASH containing 100 μL of MACS anti-FITC beads for 15 mins on ice, then washed thrice and resuspended in 10 mL of FACSWASH and applied to the LS separation columns under magnetic fields. Captured cells were eluted in the absence of the magnetic fields and collected as the T cell fraction, the purity of which was analysed using flow cytometry. In general, 2-5X10^5 T cells were seeded in 96 well culture plates in complete DMEM media and subjected to different cytokine treatments. At 72 h post treatment, the supernatants (100 μL) harvested from splenocytes cultured in different treatments were analysed for IFNγ release using ELISA based assays (BD Pharmingen).

RESULTS AND DISCUSSION:

1. CTLL-2 cells are suitable for mIL12and/ormIL18 bioassay: Since we were interested in evaluating functional murine IL12 and /or IL18 cytokines in laboratory samples, we required a T cell line of mouse origin. We used a murine cytotoxic T-cell line derived from C57BL/6 inbred mice (H-2^b) (Gillis and Smith, 1977; Baker *et al.*, 1979). These cells continuously express IL2 receptors and depend entirely the presence of IL2 for growth. They have been used for the bioassay of IL2, in both human and murine samples. We chose this cell line because i) it is of murine origin and can be maintained in culture using both human and mouse IL2 ii) it is commonly available in most immunology laboratories unlike the IL12 responsive 2D6 mouse T cell line (Maruo *et al.*, 1997), which has been successfully used to demonstrate synergies between the two cytokines (Ahn *et al.*, 1997) iii) data obtained from flow cytometric analyses of the immunostained cells showed that at least 60% of CTLL2 cell population have mIL12 receptors (Figure 1

panel A). However, when CTLL-2 cells were immunostained with ∝mIL18 receptor antibody, only ~5% of the cells had ∝mIL18 receptors (data not shown). These data suggested that CTLL-2 cells might be responsive to the stimulatory action of the cytokine mIL12, but the responses to mIL18 cytokine may be minimal. However, based on studies showing upregulation of each other's receptors as a probable mechanism to explain the established synergy between the mIL12 and mIL18 (Ahn *et al.*, 1997) we thought that a bioassay could be developed to evaluate the functionality of the two cytokines together using CTLL-2 cells. This would potentially be very useful, especially when assaying for the two cytokines in synergistic studies.

2. <u>The sensitivity of the new WST-1-based analysis of proliferation of CTLL2 cells is similar to that observed with the traditional Thymidine incorporation method</u>: Since proliferation and anti-proliferation based assays are the most commonly used bioassays for cytokines, we decided to estimate the level of cytokine through their ability to stimulate or inhibit the proliferation of the CTLL2 cells. The most commonly used method involves measuring DNA synthesis as an estimate of cell proliferation and generally determines the amount of tritiated thymidine incorporated into DNA. While this method is automated and very sensitive, it requires an elaborate set-up and the facilities to handle radioactive materials and their disposal. Alternative non-radioactive methods involve the measurement of the cellular metabolism to evaluate cell proliferation. In recent years different tetrazolium salts such as redox sensitive formazan [3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide](MTT) (Mosmann, 1983), XTT(Roehm *et al.*, 1991) or MTS (Buttke *et al.*, 1993) have been described. The salts are cleaved to formazan by cellular enzymes, and the product directly correlates to the number of metabolically active cells in culture. For this study we used a new non-

radioactive cell proliferation reagent (4-[3-(-4-Iodophenyl)-2-(4-nitrophenyl0-2H-5-tetrazolio]-1,3-benzene disulphonate] (WST-1)(Tan and Berridge, 2000) for the following reasons: 1. In contrast to MTT, it cleaves to a water-soluble formazan crystal, which can be measured directly 2. It is more stable in comparison to XTT and MTS 3. It has a wider linear range and shows accelerated color development compared to XTT. 3. The assay itself does not involve any manipulations of the treated cells (no washing , harvesting and solubilization needed) and hence is easy to conduct. 4. It is flexible as the plates can be read and returned several times to the incubator for color development. We compared the sensitivity of WST1- with [^{3}H]TdR-based analyses for evaluation of the proliferation of the CTLL2 cells. Cells ($2x10^4$) were treated with different concentrations of the cytokine IL2 for 24H. The analysis of cell proliferation at different time points using the WST1 or [^{3}H]TdR assay showed that both methods are comparable in sensitivity. The WST1 method was able to detect IL2 concentrations down to 0.19u/mL of IL2, which was very similar to what was observed with [^{3}H] Thymidine incorporation assay (Figure 2, panel A). However the background was much higher with the WST-1 assay at 24 H incubation. We then attempted to optimise the WST-1 assay to achieve better sensitivity and our data showed that almost comparable sensitivity to the thymidine incorporation method could be achieved when $5x10^3$ CTLL2 cells were incubated for 48H instead of 24H (figure 2, panel B).

3. <u>CTTL2 cells respond to the presence of recombinant mIL12 in the growth medium</u>. As the bioassay was being developed to examine the proliferative effects of mIL12 and mIL18 on CTLL2 cells, the normal doses of mIL2 (20 u/mL, Roche, USA) would have masked the effects of other cytokines. The aim of the first set of experiments was to determine a suboptimal concentration of mIL2 that would allow the cells to survive but

not to proliferate. Cells were incubated with different doses of mIL2 (50-0 units/ml) and analysed for proliferation at 24, 48 and 72 h. *These data suggested that at 2μ/L these cells were able to survive until 72 h without proliferating.* From this point on all assays were done using assay media containing mIL2 at 2μ/mL. In addition, the integrity and IL2 responsiveness of CTLL-2 cells was determined by the proliferative IL2 dose response curve in all experiments (Figure 3, panel A).

CTLL-2 cells ($5x10^3$) were incubated with recombinant mIL12 at different doses ranging from 0-100 ng/mL in the presence or absence of anti-IL12 antibody at 1μg/well. At 72 h post treatment, the cells showed a clear proliferative response to mIL12. The enhancement was greatest (7fold) for treatment with mIL12 at12.5 ng/mL (Figure 3, Panel A). This enhancement was completely obliterated in the presence of αmIL12 antibody, suggesting the specific nature of this response. When compared with the traditional splenocyte based IFNγ release assay (Figure 3, panel B) we could detect mIL12 only at 10ng/ml which was similar to the sensitivity of the CTLL-2 based bioassay.

4. <u>CTTL2 cells responded weakly to the presence of recombinant mIL18 in the growth medium</u>. Next we wanted to test the proliferative responses of CTLL-2 cells to the murine cytokine mIL18. CTLL-2 cells ($5x10^3$) in 2u/mL IL2 were incubated with recombinant mIL18 at different doses ranging from 0-100 ng/mL in the presence or absence of anti-IL18 antibody at 2μg/well (determined on the basis of personal communication Dr. Carl Power from our laboratory). At 72 h post treatment, the cells did not show any clear proliferative response to mIL18. Some enhancement was observed at the highest dose of 100 μg/mL (Figure 4). While not significant, the marginal response was obvious. This minor response could be attributed to a small percentage of mIL18

receptors expressed on these cells. This enhancement was reduced in the presence of αmIL18 antibody, suggesting the specific nature of this response.

4. CTLL2 proliferation is further enhanced when treated with both mIL12 and mIL18 in tandem.

As the proliferative response of the CTLL2 cells to mIL18 was not as strong as that observed with mILl2, this experiment was designed such that the proliferative effects of the two cytokines in combination could be assayed instead. *The rationale was that initial incubation with mIL12 would upregulate the mIL18 receptors in CTLL2 cells and this would lead to an enhanced response of the CTLL2 cells to mIL18 as a follow up treatment*. This was also supported by the observations made by Ahn *et al.*, (1997), with 2D6 cells; the cells were responsive to mIL18 only when they were pretreated with mIL12. They also showed that this was due to the increase in mIL18 receptors on these cells after mIL12 treatment (Ahn *et al.*, 1997). Cells were treated with a range of concentrations of mIL12 (0-100 ng/mL) and after 24 h incubation mIL18 was added to the cultures at varying concentrations (0-100 ng/mL). These incubations were done in the absence or presence of αmIL12 and/or αmIL18 antibodies. Cells treated with both cytokines showed enhanced proliferation compared with either alone (Figure 5). Specifically, this enhancement was significant (~2 fold) when 10 ng/mL of mIL12 and mIL18 were used. More importantly, this enhancement in proliferation was reversed when αmIL12 or αmIL18 or αmIL12+αmIL18 antibodies were used suggesting the specificity of the response to individual antibodies. While not significant, the reduction was more pronounced when the two antibodies were used together. There was some inter-assay variation. In one experiment there was up to 10 fold enhancement when 10ng/mL of mIL12 was used with 100ng/mL of mIL18. This variation could be due to

either the commonly observed variation in the responsiveness of the CTLL2 cells to the cytokines at higher passage numbers and /or due to the variation in different batches of the recombinant cytokines.

5. Evaluation of CTLL-2 based bioassay to detect and measure the functional mIL12 and/or mIL18 in biological samples.

In these experiments the aim was to assess the CTLL-2 based bioassay for determining the presence of functional mIL12 and mI18 in biological samples. For this we used supernatants from a murine prostate cancer cell line (ras-myc activated, RM1) (Thompson *et al.*, 1993) stably transformed to produce secreted mIL12 or mIL18 (RM1GFPmIL12, RM1GFPmIL18). RM1 cells stably transformed to express GFP (RM1GFP) were used as controls.

In a preliminary experiment, the CTLL-2 cells in the presence of 2 u/mL of IL2 were incubated with 2-fold serial dilutions of the supernatants for 72 h and cell proliferation was measured by WST-1 assay. CTLL-2 based bioassay successfully detected the secreted mIL12 in cell lysates from RM1-mIL12 cells, which wasn't observed when the supernatants from RM1GFP cells were used. This suggested the IL12-specificity of the bioassay. This would be further confirmed by conducting the same experiment with or without anti-mIL12 antibodies.

Experiments currently underway:

Next, we plan to evaluate the CTLL-2 cell based bioassay for evaluating functional mIL12 and mIL18 in cell lysates with predetermined (by mIL12 or mIL18 ELISA) quantities of either cytokine. The assays will be done with or without the neutralising antibodies for mIL12 and/or mIL18. We will also compare this with the traditional

bioassay by determining the capability of the cytokines to induce IFNγ release by T cells purified from splenocytes from syngeneic C57/BL6 mice.

Apart from IL2, CTLL-2 cells show a proliferative response to the cytokine IL-15 (proliferative) and anti-proliferative responses to TGF-β1 and TGF-β2 (Weller *et al.*, 1994; Chung *et al.*, 2000). It is also known that RM1 cells express functional TGFβ under certain culture conditions (Thompson *et al.*, 1992; Thompson *et al.*, 1993). Therefore, in the next set of experiments, we plan to evaluate the effects of inhibition of TGFβ activity using anti-TGFβ (neutralising) antibodies on sensitivity of this bioassay. Note: We have neutralising antibodies to TGFβ in our laboratory, which places us in an excellent position to carry out these experiments.

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FIGURE LEGENDS

Figure 1: CTLL-2 cells express mIL12 receptors: CTLL-2 cells immunostained with anti mIL12R antibody were analysed by flow- cytometry. Cells stained with the secondary antibody alone or the isotype control served as the controls. The analyses showed that 75% of CTLL2 cells express mIL12 receptor

Figure 2: Comparison of WST-1- and Thymidine incorportion-based methods for evaluation of CTLL-2 cell proliferation:$2x10^4$CTLL-2 cells were treated with the cytokine IL2 at different concentrations for 24 h and then either 10 mL of WST1 or 0.5 mci of Tritiated Thymidine were added per well. For the cells with WST-1 the absorbance was measuerd at 450 nM after 4H incubation with WST-1. The Tritiated Thymidine incorporation by the cells was measured after 8H incubation using an automated cell harvested and software "Top Count'. Panel A shows the graphs obtained after 24 H incubation of CTLL-2 cells with IL2 using the two different methods. Panel B shows the absorbance at 450 nM after $5x10^3$ CTLL-2 cells were incubated with IL2 for 48 H.

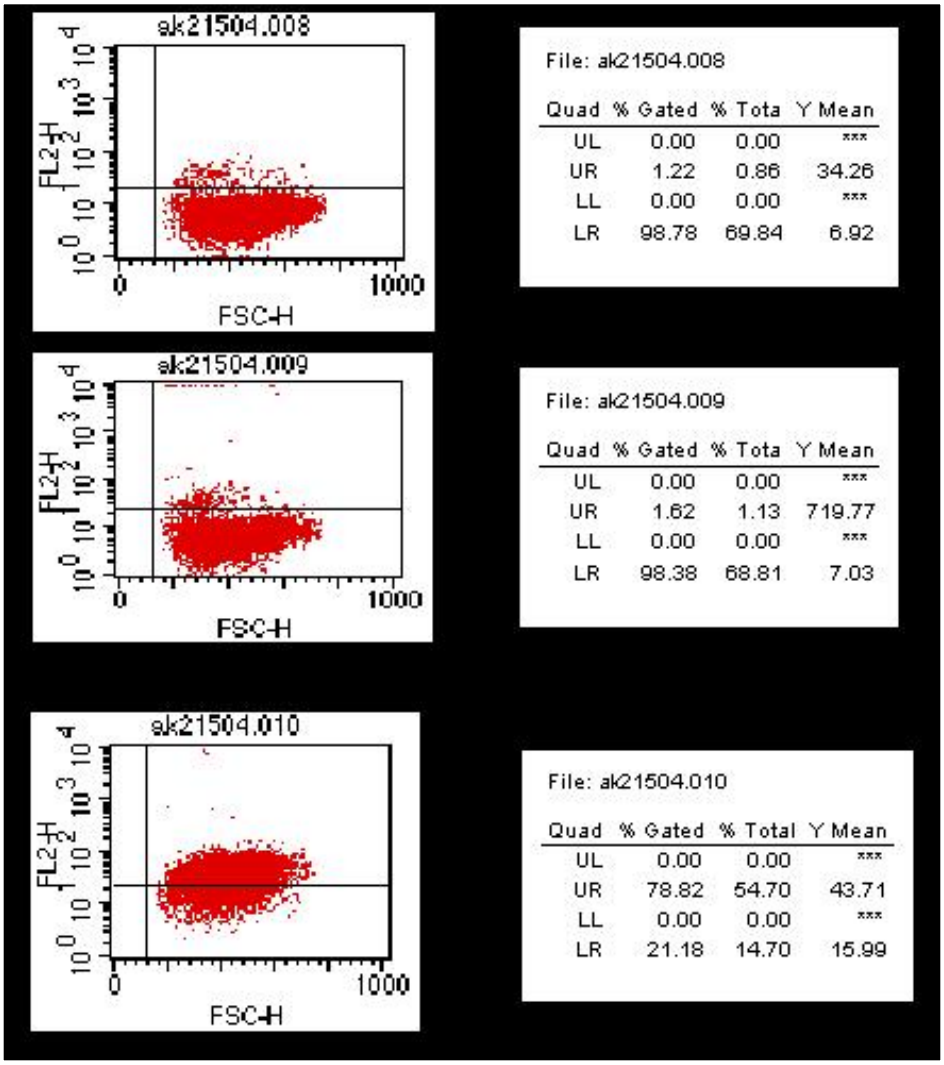
Figure 3: CTLL-2 cells proliferate in response to mIL12: CTLL-2 cells were checked for IL2 responsiveness by evaluating their response to different doses of IL2 (Panel A). To evaluate the responsiveness of CTLL-2 cells to mIL2 cells were maintained in submitotic doses of 2u/mL of IL2 as determined from their IL2 dose response curve. These were then treated with different concentrations of mIL12 with or without anti mIL12 antibodies. The second graph in Panel A shows the absorbance measured at 450nM, 72H post treatment of CTLL-2 cells with mIL12, with and without the mIL12 neutralising antibodies. Panel B shows IFNg released by purified T cells (mouse splenocytes) 66h after treatment with mIL12 with and without anti mIL12antibody.

Figure 4: Proliferation of CTLL2 cells in response to mIL18: CTLL-2 cells in 2u/mL of IL2 were treated with different doses of mIL18 with or without mIL18 neutralising antibody. Some proliferative responses could be detected at higher doses of mIL18.

Figure 5: Proliferation of CTLL-2 cells in response to combination of mIL12 and mIL18. CTLL-2 cells maintained in 2u/mL of IL2 were treated with mIL12 at different doses for 24 H and then treated with different doses of mIL18 for further 48 H. The absorbance was measured at 450nm. This repeated in the presence of anti mIl12 and/or anti-mIL18 antibodies.

Figure 6: CTLL-2 cells based bioassay could be used for detection of mIL12 in biological samples. CTLL-2 cells maintained in 2u/mL of IL2 were incubated with different % of supernatants obtained from RM1GFPmIL12 or RM1GFP cells for 72 H. The cells clearly proliferated in a dose dependant manner, when exposed to supernatants from RM1GFPmIL12 cells and this response was absent when supernatants from RM1GFp cells were used suggesting the specificity of the response.

Figure 1:



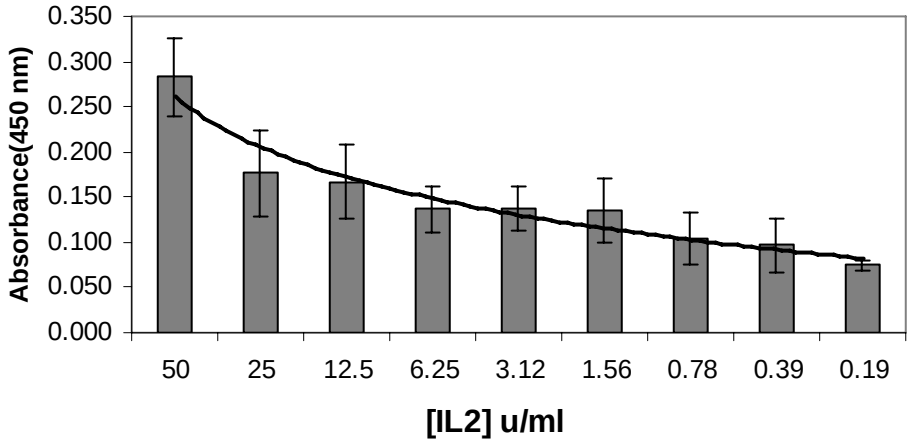
CTLL-2 cells+ Secondary antibody alone

CTLL-2 Cells + Isotype control

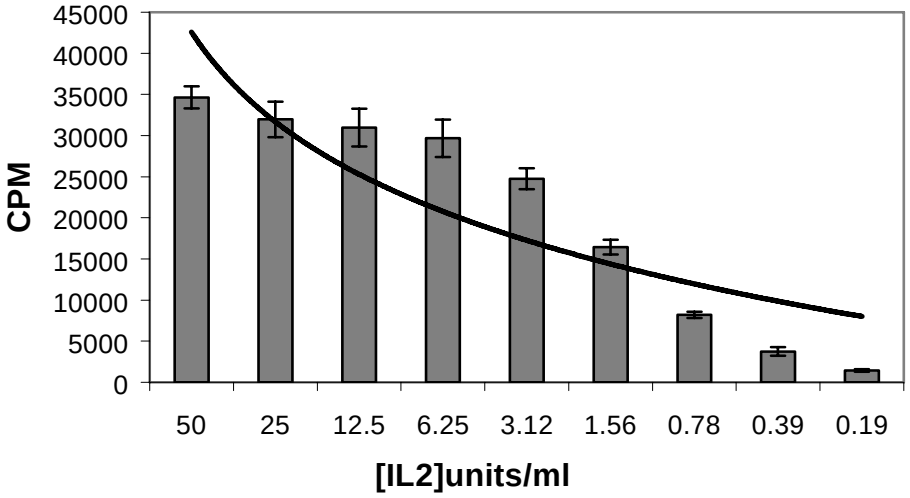
CTLL-2 Cells + anti-mIL12 receptor antibody

Figure 2

A.

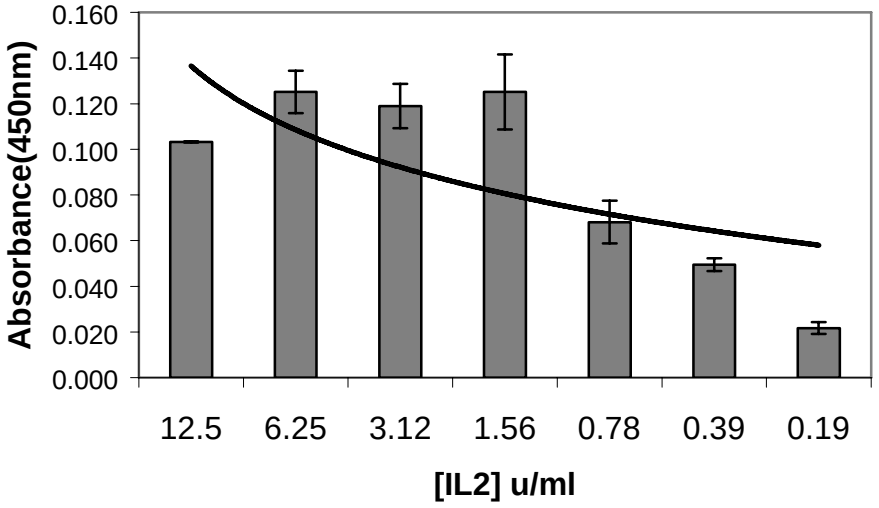


WST-1: 24H



Tritiated thymidine: 24H

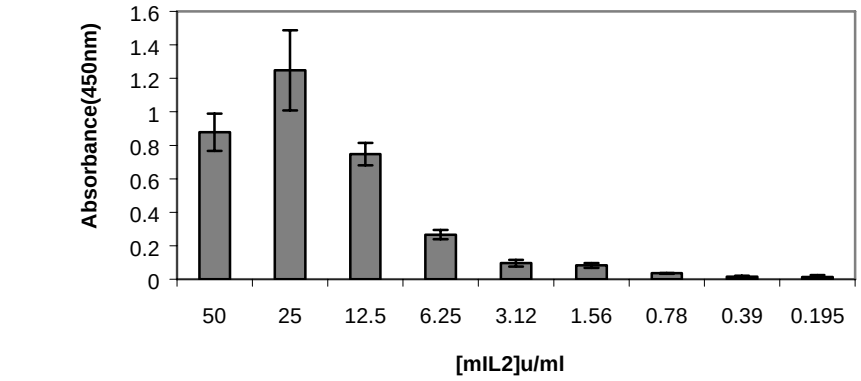
B.

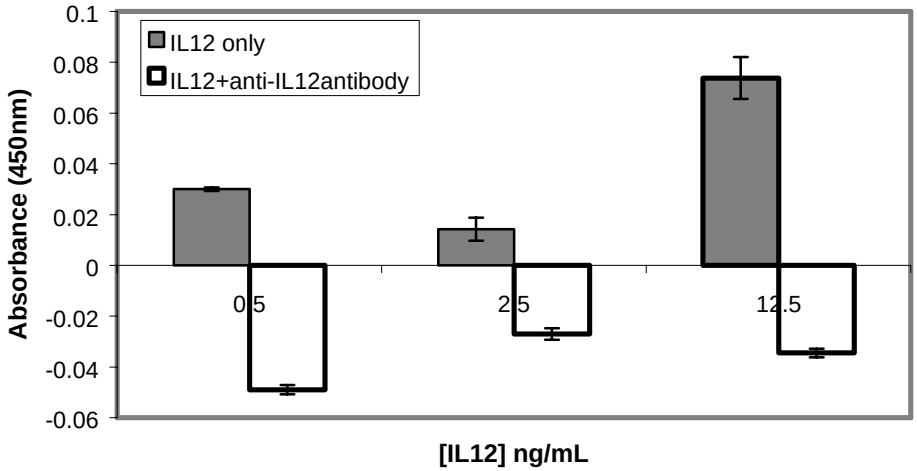


WST-1:48H

Figure 3:

A.


Absorbance(450nm)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
50
25
12.5
6.25
3.12
1.56
0.78
0.39
0.195
[mIL2]u/ml


IL12 only
IL12+anti-IL12antibody
Absorbance (450nm)
0.1
0.08
0.06
0.04
0.02
0
-0.02
-0.04
-0.06
0.5
2.5
12.5
[IL12] ng/mL

B.

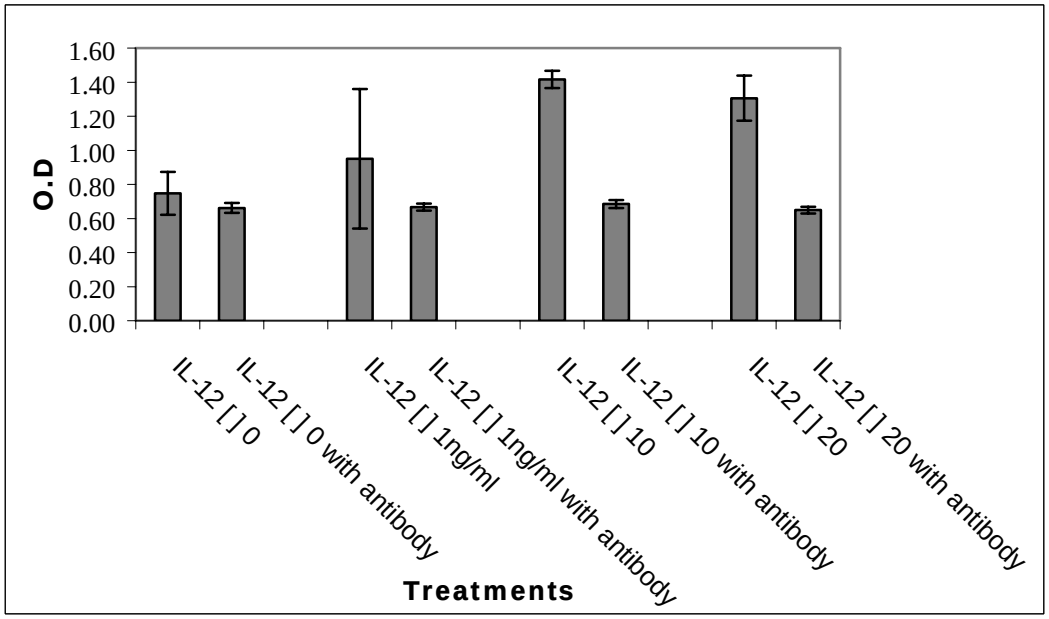

O.D
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
IL-12 [ ] 0
IL-12 [ ] 0 with antibody
IL-12 [ ] 1ng/ml
IL-12 [ ] 1ng/ml with antibody
IL-12 [ ] 10
IL-12 [ ] 10 with antibody
IL-12 [ ] 20
IL-12 [ ] 20 with antibody
Treatments

Figure 4:

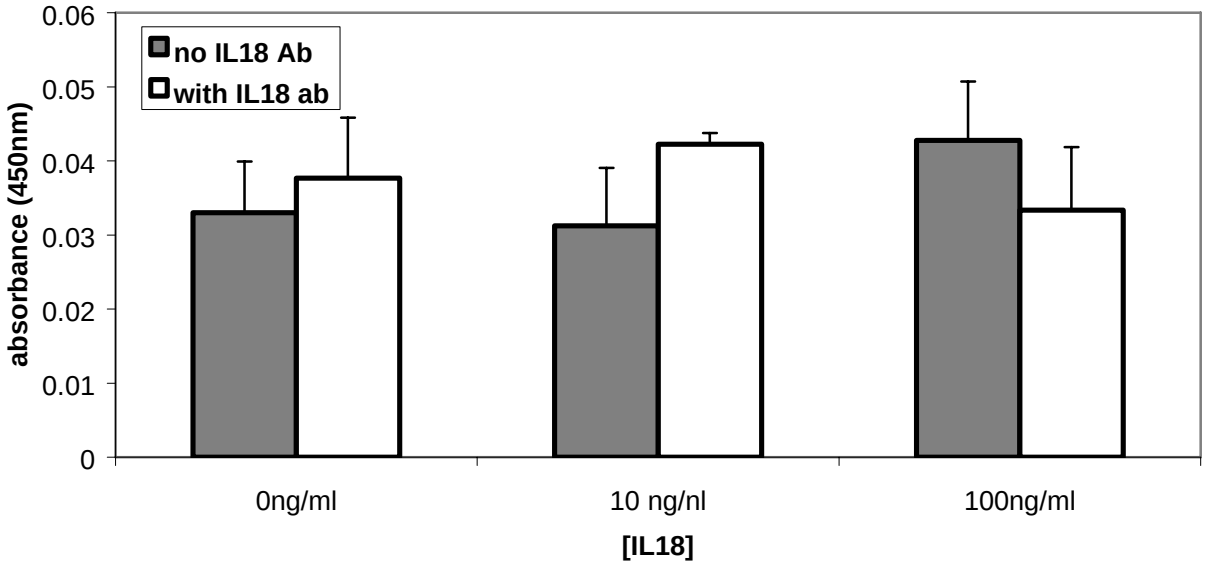


Figure 5:

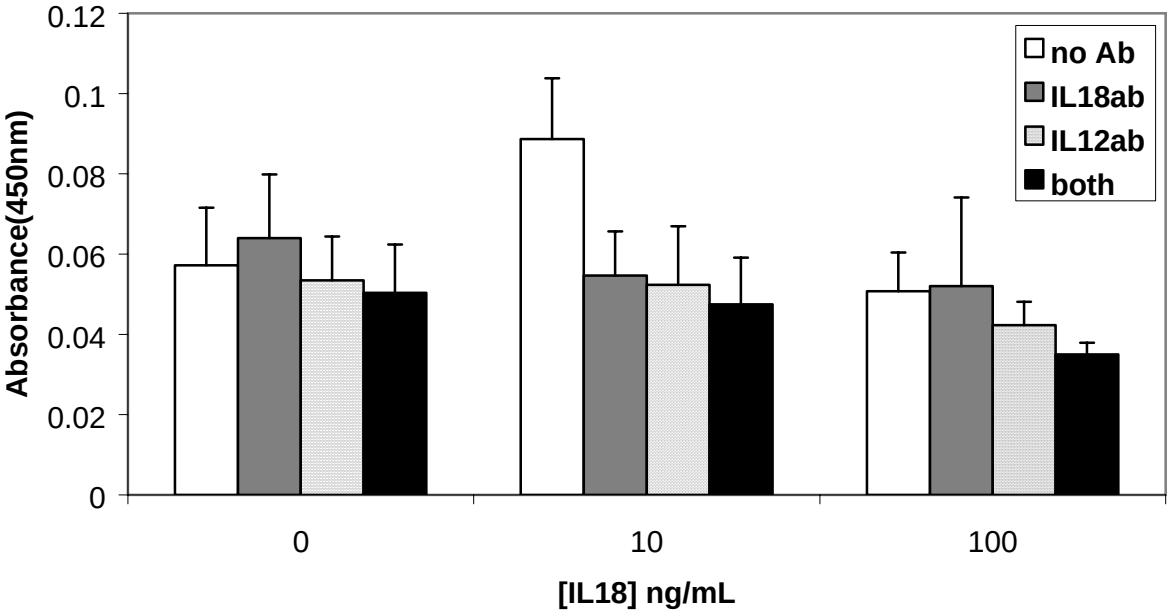
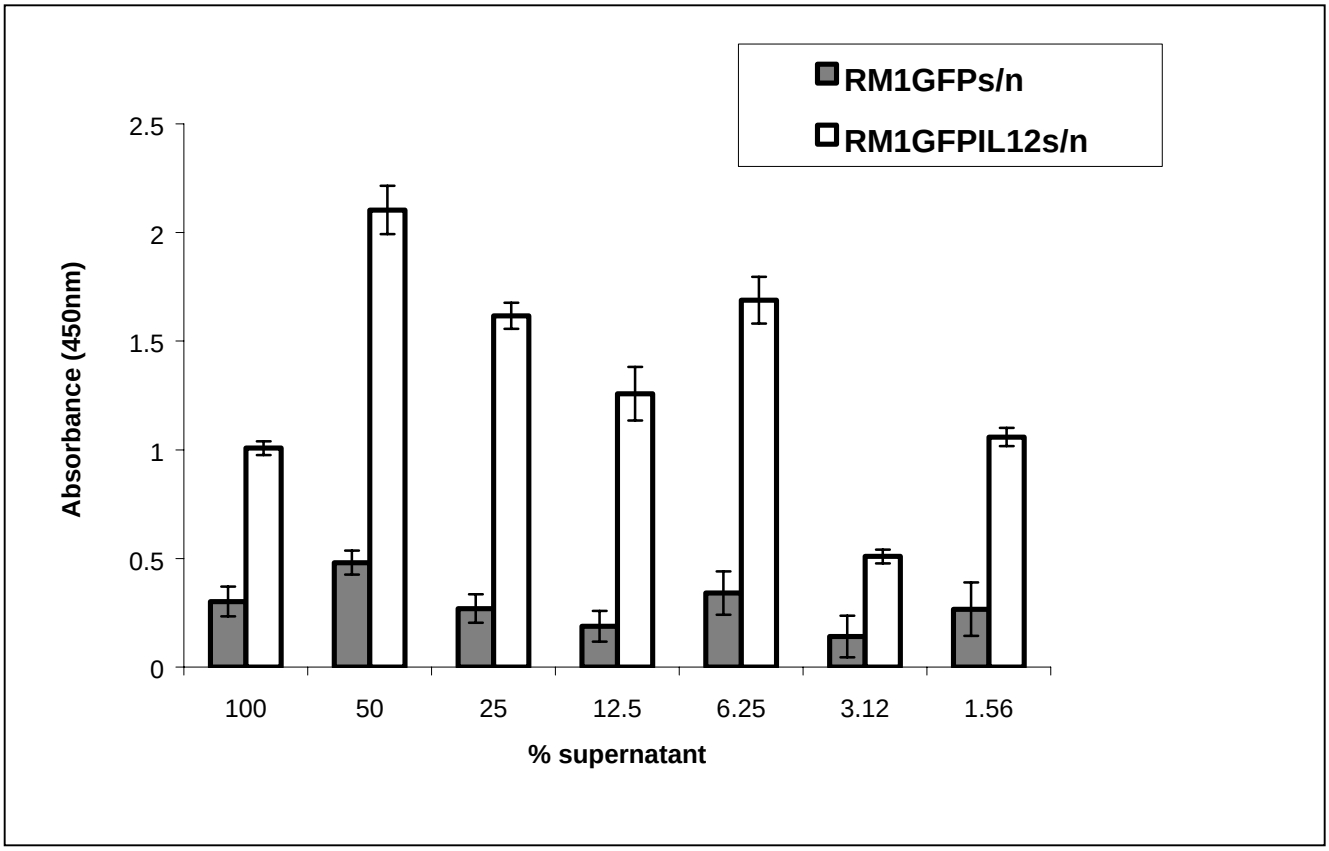


Figure 6:


RM1GFPs/n
RM1GFPIL12s/n
Absorbance (450nm)
2.5
2
1.5
1
0.5
0
100
50
25
12.5
6.25
3.12
1.56
% supernatant

Tow Prize, Prince of Wales Hospital September, 2004

GENERATION OF AN *IN VIVO* MODEL FOR EVALUATION OF PROGNOSTIC, PREVENTATIVE AND THERAPEUTIC STRATEGIES FOR LATE STAGE PROSTATE CANCER

E. Doherty, B. Curley, A. Khatri, N. Greenberg and P.J. Russell
Oncology Research Centre, Prince of Wales Hospital

Prostate Cancer is currently the most common cancer and the second highest cause of cancer death among men in Western society. Whilst early stage disease can be treated with traditional therapies (surgery, radiation and chemotherapy), metastatic prostate cancer is presently incurable. A relevant preclinical model that represents all stages of the human disease is required to evaluate potential therapies for these cancers. Although commonly used, transplantable syngeneic or xenogeneic murine models do not emulate the considerable biological and technical challenges inherent in cancer treatment. The transgenic adenocarcinoma of the mouse prostate (TRAMP) model closely mimics early stages of the human disease. However, this model does not adequately represent late stage androgen-independent, metastatic cancer.

To rectify this we have developed potentially metastatic, androgen-independent derivatives of the TRAMP C1 and C2 cell lines to broaden the TRAMP model to include cell lines indicative of the various stages of prostate cancer progression. These androgen independent cell lines were established using two different approaches, an *in vitro* hormone deprivation of TRAMP C1 and C2 cell lines, and the *in vivo* derivation of an androgen independent TRAMP C1 cell population that grew in a female mouse. Characterisation of these cell lines for various markers of androgen independent, late stage metastatic prostate cancer and determination of their *in vitro* and *in vivo* growth characteristics is currently underway. This could potentially expand the TRAMP model to represent late stage androgen independent, metastatic disease and greatly facilitate the evaluation and development of future treatments for presently incurable late stage cancers.

1. Jemal, A., Tiwari, R.C., Murray, T., Ghafoor, A., Samuels, A., Ward, E., Feuer, E.J. & Thun, M.J. (2004) *CA Cancer J Clin*. 54, 8-29

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Activity : Abstract Submission
Current Date/Time : 11/28/2004 11:32:47 PM

Gene directed enzyme prodrug therapy using the fusion gene, cytosine deaminase uracil phosphoribosyl transferase leads to a distant bystander effect in mouse models of prostate cancer.

Short Title:
CDUPRT-GDEPT for prostate cancer

Pamela J. Russell, *Aparajita Khatri*, *Bing Zhang*, *Eboney Doherty*, *Kim Ow*, *Jane Chapman*, *Rosetta Martiniello-Wilks*. Prince of Wales Hospital, Randwick, Australia, Prince of Wales Hospital, Sydney, Australia, Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia

We are evaluating the therapeutic potential of gene directed enzyme prodrug therapy (GDEPT) using the fusion gene,cytosine deaminase uracil phosphoribosyl transferase (CDUPRT) for treating prostate cancer (PCa).
Objective: To test the efficacy of CDUPRT-GDEPT against RM1 mouse androgen-refractory PCa grown in C57BL/6 mice: RM1 cells were stably transfected with green fluorescence protein (GFP) and the fusion gene, CDUPRT, derived from *E coli* (RM1-GFP/CDUPRT). **CD/UPRT converts 5 fluoro-cytosine (5FC) to freely diffusible metabolites including 5-fluorouracil (5FU), that disrupt the metabolic pathways for both DNA and RNA synthesis, thus killing both dividing and non-dividing cells.** This is especially relevant to PCa, which is characterized by a low proportion of dividing cells.
Experimental Design: RM1 cells were stably transfected with plasmids containing GFP/CDUPRT, GFP or GFP/LacZ (controls) using lipofectamine. Cells that highly expressed GFP were selected by flow cytometry and used for further study. Transgene CDUPRT expression in cell lysates from cells grown *in vitro* or after *in vivo* implantation of RM1-GFP/CDUPRT was assessed by enzymic conversion of its substrate using HPLC. To assess the local bystander effect of CDUPRT-GDEPT, C57BL/6 mice were implanted directly into the prostate with cell mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions; 4 days later, 5FC was given intraperitoneally (ip) for 13 days at 500mg/kg/mouse/day. Pseudo-metastases in the lungs were established by a tail vein injection (iv) of untransfected RM1 cells 4 days post intraprostatic implantation**.** Mice were euthanased on day 19, and prostate weight and volume, and lung weight and colony counts were assessed. Tumors, lymph nodes, spleens and lungs were frozen or fixed for immunohistochemistry.
Results: Intraprostatic RM1-GFP/CDUPRT tumors on treatment with 5FC for 13 days resulted in complete regression of the tumors. Injection of cell mixtures (RM1-GFP/CDUPRT + RM1-GFP) resulted in a local bystander effect when only 20% of

the cells were expressing the CDUPRT transgene. Interestingly, the lung colony counts indicated the presence of a distant bystander effect. The pseudo-metastases were absent in ~50% of mice in the RM1-GFP/CDUPRT+5FC group compared with the control groups. This is the first demonstration of a distant bystander effect using CDUPRT-GDEPT.
Conclusions and future work: The CDUPRT GDEPT leads to a significant local and a distant bystander effect when used to treat androgen refractory RM1 tumors in mice. The role of the immune system in this distant bystander effect is currently under investigation.
Author Disclosure Block: P.J. Russell, None; **A. Khatri**, None; **B. Zhang**, None; **E. Doherty**, None; **K. Ow**, None; **J. Chapman**, None; **R. Martiniello-Wilks**, None.

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Australasian Gene Therapy Society, Melbourne, Oct, 2005

Combination of Cytosine Deaminase with Uracil Phosphoribosyl Transferase leads to local and distant bystander effects against RM1 prostate cancer in C57BL/6 mice.

Aparajita Khatri[1,2], *Pamela J. Russell*[1,2] , *Bing Zhang*[3], *Eboney Doherty*[1,2], *Kim Ow*[1], *Jane Chapman*[1,2], *Rosetta Martiniello-Wilks*[4].

[1]Prince of Wales Hospital, Randwick, Australia,[2] University Of New South Wales, Sydney, [3]University of Queensland, Brisbane, [4]Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia

We are evaluating the therapeutic potential of gene directed enzyme prodrug therapy (GDEPT) using cytosine deaminase (CD) in combination with uracil phosphoribosyl transferase (UPRT) for treating prostate cancer (PCa). *CDUPRT converts 5 fluoro-cytosine (5FC) to freely diffusible metabolites, including 5-fluorouracil (5FU), that disrupt the metabolic pathways for both DNA and RNA synthesis, resulting in the killing of both dividing and non-dividing cells*. This is especially relevant to slow growing PCa. Andogen-independent mouse RM1 cells were stably transformed with plasmids containing GFP/CDUPRT, GFP or GFP/LacZ genes (controls). CDUPRT expression in cell lysates from RM1-GFP/CDUPRT cells/tumors was confirmed by estimation of enzymic conversion of its substrate, 5FC to 5FU using HPLC. Treatment of C57BL/6 mice bearing intraprostatic RM1-GFP/CDUPRT tumors with 5FC resulted in complete regression of the tumors. Further, intraprostatic implantations with mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions in C57BL/6 mice resulted in a '***local bystander effect',*** even though only 20% of the cells were expressing the transgene. To determine if there was any distant bystander effect, pseudometastases in the lungs were established and the lung colony counts at necroscopy (day 19) indicated the presence of a '***distant bystander effect***'. Indeed, the pseudometastases were absent in ~50% of mice in the RM1-GFP/CDUPRT+5FC group compared with the control groups. ***This is the first demonstration of a distant bystander effect using CDUPRT-GDEPT.*** Furthermore, immunohistochemical evaluation of the GDEPT showed an increase in immune cell infiltration by $CD4^{+}$ T cells, macrophages and natural killer cells. There was increased tumor necrosis and apoptosis and a decrease in tumor vascularity after GDEPT. We conclude that CDUPRT-GDEPT significantly suppressed the aggressive growth of RM1 prostate tumors via mechanisms involving necrosis and apoptosis, accompanied by strong infiltration of immune cells in the prostate tumors. The latter may be associated with the decrease in lung pseudometastases.

Australian Prostate Cancer Collaboration Annual Meeting, September, 2005, Sydney, Australia

COMBINED MOLECULAR CHEMOTHERAPY AND CYTOKINE MEDIATED IMMUNOTHERAPY FOR THE TREATMENT OF PROSTATE CANCER

Aparajita Khatri[1,2], *Bing Zhang*[3], *Eboney Doherty*[1,2], *Kim Ow*[1], *Hnin Pwint*[3], *Jane Chapman*[1,2], *Rosetta Martiniello-Wilks*[4] *and Pamela J. Russell*[1,2]

[1]Oncology Research Centre, Prince of Wales Hospital, Randwick, Australia,[2] Department of Medicine, University Of New South Wales, Sydney, [3]University of Queensland, Brisbane, [4]Centenary Institute of Cancer Medicine & Cell Biology, Sydney, Australia

Supported by US Army Medical Research and Material Command (Award No: DAMD)

Introduction: We are evaluating the therapeutic potential of combination therapy involving gene directed enzyme prodrug therapy (GDEPT) using cytosine deaminase-uracil phosphoribosyl transferase (CDUPRT) with murine IL18- and IL12-mediated immunotherapy for treating prostate cancer (PCa).

Methods: Stably transformed andogen-independent mouse PCa cells, RM1-CDUPRT, -GFP or GFP/LacZ cells were used. To assess the local bystander effects of CDUPRT-GDEPT, immunocompetent C57BL/6 mice implanted with cell mixtures of RM1-GFP/CDUPRT and RM1-GFP cells in different proportions intra-prostatically were treated with 5 fluorocytosine (5FC). Pseudo-metastases in the lungs were established by a tail vein injection of untransfected RM1 cells. At necroscopy, prostate weight/volume and lung colony counts were assessed. Tumours, lymph nodes, spleens and lungs were frozen or fixed for immunohistochemistry. For combination studies, mice with RM1-CDUPRT tumours were injected with plasmids encoding either mIL12 or mIL18 or a combination of both, intraprostatically.

Results: Treatment of mice bearing intraprostatic RM1-GFP/CDUPRT tumors with 5FC resulted in complete regression of the tumors. Further, a '***local bystander effect'*** was demonstrated by tumor regression even though only 20% of the cells were expressing the transgene. Significant reduction in pseudo-metastases of RM1 cells in lungs of GDEPT-treated mice indicated a ***'distant bystander effect'***. The GDEPT-treated tumours showed increased necrosis and apoptosis, with decreased tumour vascularity. There was a significant increase in tumour-infiltration by macrophages, $CD4^+$ T and natural killer cells. Finally, GDEPT in combination with either mIL12 or mIL18 led to a significant reduction in lung psuedometastases compared with either modality alone.

Conclusions: CDUPRT-GDEPT significantly suppressed the aggressive growth of RM1 prostate tumors via *local and distant bystander effects*. The anti-tumour immune responses implicated in the latter were further augmented by combination with mIL12 or mIl18 mediated immunotherapy.

THE UNIVERSITY OF
NEW SOUTH WALES

STATEMENT OF INCOME AND EXPENDITURE
FOR THE PERIOD
1 October 2005 TO 31 December 2005

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CLIENT SERVICES
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TITLE: US ARMY RESEARCH OFFICE DAMD 17-02-1-0107
Prostate Cancer Research Program (PCRP) Formulated Delivery of Enzyme Pro-Drug & Cytokine gene therapy...
Prof PJ Russell, Dr A Khatri

PROJECT NO: RM00750

Life to Date $		$	Current Period $
0.00	**OPENING BALANCE UNSPENT/(OVERSPENT)**		**292,942.42**
	OPERATING INCOME		
813,957.80	Grant received	0.00	
0.00	Miscellaneous	0.00	
813,957.80	* **TOTAL OPERATING REVENUE**		**0.00**
	OPERATING EXPENDITURE		
469,051.82	Salaries	76,188.71	
18,451.44	Equipment	0.00	
121,706.01	Materials	13,687.05	
0.00	Scholarships	0.00	
0.00	Contract services	0.00	
4,813.87	Travel	3,132.00	
0.00	Research Infrastructure charge	0.00	
614,023.14	**TOTAL OPERATING EXPENDITURE**		**93,007.76**
199,934.66	**CLOSING BALANCE UNSPENT/(OVERSPENT)**		**199,934.66**
	COMMITMENTS OUTSTANDING AT PERIOD END		
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0.00	Materials	0.00	
0.00	Scholarships	0.00	
0.00	Contract services	0.00	
0.00	Travel	0.00	
0.00	Research Infrastructure charge	0.00	
0.00	TOTAL COMMITMENTS OUTSTANDING		0.00

CERTIFIED CORRECT

John Cope, ASA
Senior Accountant, Research
Accounting Services
27-Jan-06

* Where GST has been received in addition to the grant funds above, the GST has been forwarded to the Australian Taxation Office.

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